NOVEMBER - DECEMBER, 1961
Volume 26, Number 6

JOURNAL OF
FOOD SCIENCE

(formerly FOOD RESEARCH)

An Official Publication of
THE INSTITUTE OF FOOD TECHNOLOGISTS

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FOOD SCIENCE

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Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 510-522 N. Hickory Street, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

NOVEMBER-DECEMBER, 1961 **Volume 26, Number 6**

An Official Publication of the Institute of Food Technologists

Food Technology

Published Monthly by the

INSTITUTE OF FOOD TECHNOLOGISTS

The Companion Journal to

FOOD SCIENCE

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THE GARRARD PRESS

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CHAMPAIGN, ILLINOIS

Journal of FOOD SCIENCE

(formerly FOOD RESEARCH)

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The Volatile Constituents of Passion Fruit Juice[a]

DAWES N. HIU[b] AND PAUL J. SCHEUER
Department of Chemistry, University of Hawaii, Honolulu 14, Hawaii

(Manuscript received March 29, 1961)

SUMMARY

The characteristic flavor of passion fruit was found in the water-insoluble oil, which constituted *ca.* 36 ppm of the juice. Four components, *n*-hexyl caproate, *n*-hexyl butyrate, ethyl caproate, and ethyl butyrate, made up *ca.* 95% of the oil, and, among these four, *n*-hexyl caproate was found to be the principal component, accounting for *ca.* 70% of the volatile passion fruit essence.

The unidentified portion of the oil (*ca.* 5%) very likely contains compounds of greater structural complexity than those identified. Evidence for this assumption comes from the appreciable specific rotation of the crude oil. It is also reasonable to assume that the olefinic unsaturation encountered in the crude oil (Table 1) has its origin in the unidentified portion of the oil.

Gas-liquid partition chromatography has become a powerful research tool for the investigation of volatile plant constituents since the method was developed by James and Martin (1954), in 1952. An example of a recent application to the study of a fruit essence is the work on strawberries by Corse and Dimick (1958).

Research on the constituents of tropical fruits has been largely confined to nutritional aspects, and generally restricted to gross analyses of sugars, vitamins, etc. A notable exception is pioneering work by Haagen-Smit *et al.* (1945) on the volatile constituents of pineapple.

The growing and processing of passion fruit *(Passiflora edulis)* have assumed importance in recent years in Australia, New Zealand, South Africa, and Hawaii. Most of the edible species of the genus *Passiflora* are endemic to tropical America. The first seeds of *P. edulis* Sims, which bears purple fruit, were introduced to Hawaii from Australia about 1880 and were planted in the Lilikoi district of East Maui (Anonymous, 1956). This circumstance has resulted in the trivial name *lilikoi* for passion fruit in Hawaii. The variety grown commercially in Hawaii is not the one that bears purple fruit, but a yellow-fruited variety, *P. edulis* f. *flavicarpa* Degener. *P. edulis* is still a common vine in the mountains of most of the islands in the Hawaiian chain. Prior research on the constituents of passion fruit have been nutritionally oriented studies in India. That work has been summarized (Pruthi and Lal, 1959).

In view of our lack of knowledge of the volatile constituents of *P. edulis*, a fruit with a unique and pleasant flavor, a study of this problem seemed desirable.

EXPERIMENTAL METHODS

Isolation of the essence. Fresh passion fruit from the University of Hawaii Experiment Station farm at Waimanalo, Oahu, was graded to remove unripe, decayed, and damaged fruit. It was then stemmed and washed, and sliced by a rotary slicer. The rind was removed by a centrifugal separator. The seeds and other solid particles were removed by two pulping machines, first to pass a 7.9-mm screen, then a 60-mesh screen.

The resulting juice was diluted 1:2 in water and processed through a fruit-essence recovery unit of the flash-heater-vaporizer-concentrator type described by Walker and Patterson (1955). An oily layer possessing the characteristic passion fruit odor separated above the steam distillate. The aqueous condensate was returned to a reflux column above a reboiler. The stripped material was checked by periodic taste tests.

[a] From the Ph.D. thesis of Dawes N. Hiu, University of Hawaii, 1959.

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The aqueous distillate was extracted with petroleum ether (b.p. 35–43°), which had been purified by shaking with concentrated sulfuric acid, washing with water, drying, and fractionating. The efficiency of this extraction was demonstrated by continuous extraction of the remaining aqueous phase with diethyl ether for 24 hours. The ether extract contained only a trace amount of residue without the characteristic passion fruit odor.

The petroleum ether extract was dried over anhydrous sodium sulfate, and the solvent was removed by distillation through a 1.8 × 25-cm column packed with glass helices. A yellow oil remained.

Examination of the organic phase. The fragrant yellow oil was subjected to the standard qualitative tests. The results are summarized below.

Gas-liquid partition chromatography (GLPC) was used in order to determine the complexity of the oil. The apparatus was built into a Cenco Dekhotinsky constant-temperature oven. The detection device was a Gow-Mac Pretzel heated filament type. The electronic circuitry of the bridge was essentially as described by Dimbat *et al.* (1956), and the sample introduction system as reported by Tenney and Harris (1957). The column support was Johns-Manville C-22 fire brick (40-60-mesh) treated with concentrated hydrochloric acid to remove iron. Two partitioning agents were used, silicone and Carbowax 1500, 4 g in each case for 10 g of fire brick. For the silicone column, Dow-Corning silicone high-vacuum grease was dissolved in ethylene dichloride with heating, and the fire brick was added with stirring. The mixture was heated on a steam bath, with occasional stirring, to remove most of the solvent. It was then dried overnight at 100°. The material was packed into 10 ft of copper tubing, 5/16-in. diameter, by use of a vibrator. The column ends were plugged with Pyrex wool, and the column was coiled to fit the oven. The Carbowax 1500 (Union Carbide and Chemical Co.) column was constructed similarly. Column temperatures were 184, 143, and 100°. The carrier gas was helium flowing at 45 ml/min. The columns were calibrated at the three operating temperatures with series of known compounds.

Quantitative estimates of the percentage composition of the oil were obtained as follows. The GLPC apparatus was calibrated with known standard mixtures. The areas under the component peaks were cut out and weighed. The areas were plotted against percentage composition. The areas under the peaks of chromatograms of the volatile oil were then compared with the standard.

Preliminary GLPC runs of the oil indicated the presence of one major and at least four minor components. Subsequent separation was fashioned accordingly, and a combination of reduced pressure distillation and column chromatography led to the desired results.

Reduced pressure distillation under a nitrogen atmosphere in semi-micro equipment (Synthetical Laboratories, Chicago, Ill.) allowed clean separation of the major component, which was identified by standard analytical methods and by synthesis.

Column chromatography was carried out in dry-packed Florisil (Floridin Co., Tallahassee, Fla.) columns (18 mm × 38 cm). The successive eluants were petroleum ether (b.p. 30–43°), diethyl ether, and acetone.

The minor components were identified by paper chromatography and spectral analysis. Infrared spectra were determined with a Beckman IR-2A, and ultraviolet spectra with a Beckman DU, instrument.

Paper chromatography was carried out on Whatman No. 1 paper in two solvent systems. *n*-Butanol-glacial acetic acid-water (40:10:50) and benzene-glacial acetic acid-water (100:75:100) were used with upward irrigation and overnight equilibration. The chromatograms were dried after the solvent front had traveled 20 cm. Comparison of the spots was carried out as described by Meigh (1955).

RESULTS AND DISCUSSION

Over-all yield of water-insoluble yellow oil was 36 ppm: In 1957, 556 kg of raw juice yielded 24.05 g of oil (43 ppm), and in 1958, 267 kg of juice furnished 6.2 g (23 ppm). The two oils were identical in composition as demonstrated by GLPC.

Table 1 shows the results of standard qualitative tests. Density of the oil was found to be $d_4^{26°} = 0.838$, and optical rotation of the pure oil $[\alpha]_D^{26°} = 4.17 \pm 0.02°$. The UV spectrum lacked complexity, exhibiting a maximum of low intensity at 280

Table 1. Qualitative tests on the volatile oil of passion fruit juice.

Test	Result	
Solubility	v.s. diethyl ether, chloroform, benzene, MeOH	
Elemental analysis	halogen	−
	nitrogen	−
	sulfur	−
Functional group analysis	carbonyl	−
	OH	−
	ester	+
	acid	−
	olefin	+

mμ, a minimum at 266 mμ in addition to end absorption. The IR spectrum had major bands at 2.89, 3.45, 5.75, 6.83, 7.25, 8.01, 8.50, 9.09, 11.09, and 13.7 μ. GLPC analysis in two systems showed five clearly defined fractions.

The major component. Vacuum distillation initially yielded four fractions. The material up to 30°/0.4 mm represented high-boiling components of the extraction solvent (petroleum ether). Fractions 3 and 4 were redistilled and cut into 3 fractions. The highest boiling fraction, 61–62°/0.2 mm, was shown by GLPC to be essentially pure. This compound had the following properties: $d_4^{20°} = 0.8707$, $n_D^{20°} = 1.4282$, $[\alpha]_D^{26°} = 0°$. All but two IR bands of the crude oil (2.89 and 7.25 μ) were present in the spectrum of this component: 3.44, 5.77, 6.81, 8.04, 8.52, 9.05, 9.90, and 13.7 μ. The new band at 9.90 μ was unresolved in the spectrum of the crude oil. The structure of this component, which was shown by GLPC to be the major constituent of passion fruit oil, was proved as follows:

1) Combustion analysis (performed by Dr. A. Bernhard, Mülheim/Ruhr, Germany) gave the following results. Calculated for $C_{12}H_{24}O_2$: C, 71.95; H, 12.08; O, 15.98; C-methyl, 15.03. Found: C, 71.54, 71.84; H, 11.55, 11.71; O, 16.62, 16.77; C-methyl, 0.45.

2) The compound was reduced by lithium aluminum hydride to an oil that lacked the carbonyl band at 5.77 μ and exhibited a new band at 3 μ. The 3,5-dinitrobenzoate of this alcoholic material was prepared in the usual manner and furnished a *single* 3,5-dinitrobenzoate, m.p. 56–57°, after several recrystallizations from MeOH–water. Analysis: Calculated for $C_{13}H_{16}N_2O_6$: C, 52.70; H, 5.44; N, 9.46. Found: C, 52.68, 52.72; H, 5.46, 5.44; N, 9.21, 9.45.

3) The major component was hydrolyzed by 1*N* potassium hydroxide in boiling ethylene glycol. The resulting acid and alcohol were isolated. An anilide of the acid had m.p. 92–94°, compared with the literature value (Vogel, 1951; p. 361) of 95° for caproic acid anilide. A 3,5-dinitrobenzoate of the alcohol melted at 57–58°, compared with 61° (Vogel, 1951; p. 267) for *n*-hexyl-3,5-dinitrobenzoate. Infrared spectra of the hydrolysis products were identical in all respects to those of caproic acid and of *n*-hexyl alcohol.

4) *n*-Hexyl caproate was synthesized from caproyl chloride and hexyl alcohol. The resulting ester was purified by repeated distillation *in vacuo* until the cut, b.p. 73–74°/0.3 mm, was shown to be pure by GLPC. Infrared and NMR spectra of synthetic *n*-hexyl caproate were identical in all respects to those of the major component.

The only anomalous—and very puzzling—result in the entire structure proof was the essentially zero value in the C-methyl determination of a compound that has *two* such groups. This result lent support to an early working hypothesis that the major component was a lactone rather than an ester. Subsequent experimental results *(vide supra)* made this hypothesis untenable. In order to correct or confirm the early analytical results, samples of natural and synthetic *n*-hexyl caproate were subjected to C-methyl determination with the following results:

	Natural		Synthetic	
% C-methyl found:	0.75,	1.13%	0.0,	0.0%
Calculated for 2 C-methyl:		15.03%		

It is therefore apparent that *n*-hexyl caproate is indeed resistant to oxidation under the conditions of the Kuhn-Roth determination and gives anomalous results.

The minor components. *Initial separation.* A crude separation was achieved by column chromatography on Florisil. Five grams of volatile oil yielded: 1) 4 g of colorless petroleum ether eluate possessing an odor very similar to that of *n*-hexyl caproate; 2) 0.78 g of straw-yellow diethyl ether eluate having an odor strongly reminiscent of peaches; and 3) 0.01 g of dark-yellow viscous oil eluted with acetone having an odor not unlike that of apricots. No GLPC plot could be obtained for the acetone eluate since the oil was too viscous to be introduced satisfactorily. Fig. 1 compares GLPC plots of the crude oil, the petroleum ether eluate, and the diethyl ether eluate. The petroleum ether fraction apparently had a composition very similar to that of the crude oil, and the diethyl ether eluate contained components not previously encountered. All identifiable

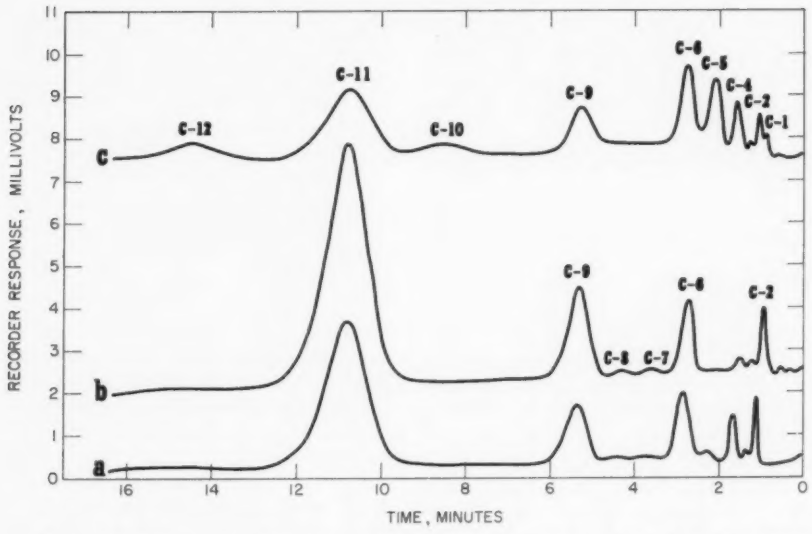

Fig. 1. GLPC's. (a) volatile oil; (b) petroleum ether eluate; (c) ethyl ether eluate. Silicone on fire brick; 184°; helium at 45 ml/min.

peaks are numbered successively with increased retention time, and are referred to by these designations hereinafter.

Identification. Since ethyl alcohol had been identified as a component of the aqueous phase (unpublished data) and *n*-hexyl caproate as the major constituent of passion fruit oil, it seemed reasonable to suspect the presence of other ethyl and hexyl esters. The relation of retention time to boiling point was therefore determined for a series of authentic esters under our experimental conditions. The results are in Table 2. To allow boiling-point assignments to be made on the basis of retention times, the above data were plotted. It should be pointed out that boiling points corresponding to very brief retention times (one minute or less) are not reliable, since the respective compounds are eluted very nearly as fast as it takes for the carrier gas to pass through the column. For very long retention times the accuracy of the estimated boiling points diminishes, since diffusion effects tend to broaden the GLPC peaks. This is particularly apparent with fractions in very low concentration, which give rise to broad peaks of small deflection. Table 3 shows retention times of component peaks and estimated boiling points.

Petroleum ether eluate. Fig. 1 indicates that the column fraction eluted with petroleum ether consisted of four principal components, C-11, C-9, C-6, and C-4, corresponding to the major constituents of the crude oil.

Component C-11, having a retention time of 10.8 min, was separated by GLPC and collected. It had a refractive index of 1.4237 (24°), compared to a value of 1.4282 (20°) determined earlier for *n*-hexyl caproate. The infrared spectra of C-11 and *n*-hexyl caproate were identical in all respects.

Table 2. Retention times of six known esters on a silicone gas-liquid partition chromatography column at 184°.

Ester	Retention time (min)	Boiling point (° C)
Ethyl acetate	1.14	77
Ethyl butyrate	1.64	121
Ethyl caproate	2.82	168
n-Hexyl acetate	2.84	169
n-Hexyl butyrate	5.34	208
n-Hexyl caproate	10.8	245

Table 3. Retention times and estimated boiling points of all component peaks on a silicone gas-liquid partition chromatography column at 184°.

Component	Retention time (min)	Estimated boiling point (° C)
C-1	0.96	
C-2	1.12	77
C-3	1.36	96
C-4	1.64	120
C-5	2.16	145
C-6	2.82	170
C-7	3.60	185
C-8	4.40	198
C-9	5.34	208
C-10	8.66	238
C-11	10.80	245
C-12	14.44	
C-13	16.5	
C-14	23	

Component C-9, with a retention time of 5.34 min, corresponded to a compound having a b.p. *ca.* 208°. Its identity was proved by comparison with an authentic sample of *n*-hexyl butyrate. Its refractive index was 1.4165 (20°), compared to a literature value (Huntress and Mulliken, 1941) of 1.4188 (15°); paper chromatography of its hydroxamic acid derivative showed it to be an ester of butyric acid; and its infrared spectrum was identical in all respects to that of a synthetic sample, b.p. 52–53°/0.5 mm, n_D^{26} 1.439.

Component C-6, retention time 2.8 min, corresponded to a compound having a b.p. *ca.* 167°. The material was separated by GLPC and collected. A paper chromatogram of the hydroxamic acid derivative in BuOH-AcOH-water exhibited two spots, R_f 0.83 and 0.08, respectively corresponding to caproic and acetic acids. An infrared spectrum of this component was identical with the spectrum of ethyl caproate, with one deviation: our compound had at 12.4 μ a band that was lacking in the spectrum of ethyl caproate. Component C-6 therefore represents slightly impure ethyl caproate.

To allow identification of C-4 the GLPC apparatus was recalibrated at 143°. Standard retention times were found in the manner described above for the higher temperature. In this manner C-4 was identified as ethyl butyrate, and the identity of C-6 as ethyl caproate was confirmed.

Diethyl ether eluate. Further gross separation of this material into two fractions was obtained by rechromatography on Florisil and elution with petroleum ether-diethyl ether (1:1). Decreasing amounts of yellow oil were eluted in fractions 1–7 and a viscous yellow oil of different odor in fractions 8–10. The first seven fractions were combined, rechromatographed on Florisil, developed with petroleum ether, and eluted with increasing concentrations of diethyl ether. GLPC analysis of the resulting 24 chromatographic fractions yielded the following information: Fractions 1–4 consisted essentially of solvent; fractions 5–8 consisted of a mixture of C-4, C-6, C-9, and C-11, which had been previously encountered in the petroleum ether eluate and identified. None of the remaining fractions were obtained in sufficient amount for positive identification. Spectral data were obtained of several fractions having the characteristic odor of roses, apricots and peaches.

QUANTITATIVE ESTIMATES OF THE IDENTIFIED COMPONENTS

Three standard mixtures of *n*-hexyl caproate, *n*-hexyl butyrate, ethyl caproate, and ethyl butyrate, corresponding to C-11, C-9, C-6, and C-4, were prepared in proportions similar to those found in the passion

Table 4. Composition of quantitative standards for estimation of C-4, C-6, C-9, and C-11.

Component	Standard 1			Standard 2			Standard 3		
	Wt (g)	% wt	% area	Wt (g)	% wt	% area	Wt (g)	% wt	% area
Ethyl butyrate C-4	0.0774	7.70	9.4	0.0484	4.81	6.9	0.0305	3.04	4.7
Ethyl caproate C-6	0.1289	12.8	14.0	0.1061	10.6	11.0	0.0683	6.80	8.2
n-Hexyl butyrate C-9	0.1989	19.8	19.1	0.1482	14.8	14.8	0.0983	9.79	10.6
n-Hexyl caproate C-11	0.6000	59.7	57.6	0.7023	69.9	67.3	0.8067	80.4	76.5

Table 5. Weight percent composition of four principal components of volatile passion fruit oil.

Component	1957 Oil			1958 Oil		
	Wt of peak (g)	% of total area	% composition	Wt of peak (g)	% of total area	% composition
Ethyl butyrate C-4	0.0023	3.3	1.0	0.0028	3.7	2.0
Ethyl caproate C-6	0.0087	12.4	11.6	0.0074	9.7	9.0
n-Hexyl butyrate C-9	0.0101	14.4	14.2	0.0118	15.5	15.2
n-Hexyl caproate C-11	0.0492	70.0	73.0	0.0543	71.0	74.0

fruit oil. The data are in Table 4. When known percentage composition was plotted against corresponding areas under the peaks, straight lines were obtained for the three experimental points in all cases. The plots are reproduced in Fig. 2. A GLPC trace of the total volatile oil was used to determine the relative areas under the peaks representing C-4, C-6, C-9, and C-11 by cutting out the peaks and weighing the paper. Percentage composition was then read from the calibration curves (Fig. 2). These data are in Table 5. The peaks C-1 and C-2, which had been shown to be caused by petroleum ether, were neglected, and all remaining peaks were weighed. Comparison of this weight with the weight of peaks C-4, C-6, C-9, and C-11 showed that the four identified components represented 94.7% of the total volatile oil. The percentage figures arrived at in Table 5 were then corrected for this factor. The true percentage composition by weight is shown in Table 6.

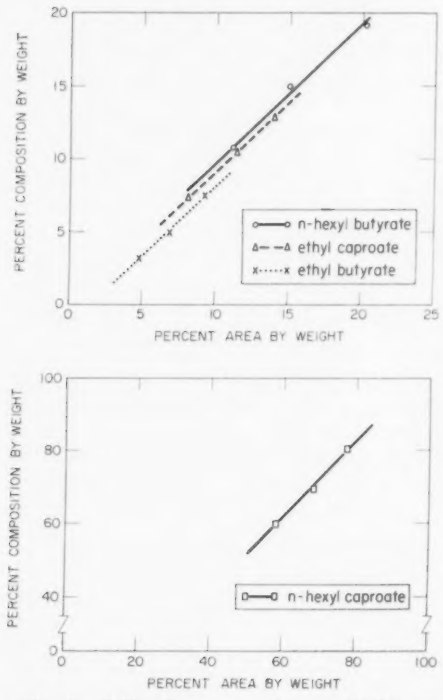

Fig. 2. Calibration curves for quantitative analyses of components C-4, C-6, C-9, and C-11.

DISCUSSION

It is interesting to compare these results with some of those reported for other fruits. The volatile oil of banana (13 ppm) is composed largely of amyl esters of acetic, isovaleric, isocaproic and caprylic acids in addition to a number of unidentified compounds (Haagen-Smit *et al.*, 1945); grapefruit essence (15.7 ppm) is made up largely of limonene (Kirchner and Miller, 1953); and strawberry oil (75 ppm) contained over 40 compounds of which *ca.* 20 have been identified. The esters among the identified compounds were ethyl butyrate, isovalerate, and caproate, as well as isoamyl and *n*-hexyl acetates (Corse and Dimick, 1958). To our knowledge this is the first report of the occurrence of *n*-hexyl caproate in a natural product. The component alcohol and acid

Table 6. Percent composition by weight of volatile passion fruit oil.

Component	Weight percent composition	
	1957	1958
Ethyl butyrate C-4	0.95	1.9
Ethyl caproate C-6	11.0	8.5
n-Hexyl butyrate C-9	13.4	14.4
n-Hexyl caproate C-11	69.1	70.1
Unidentified components	5.55	5.1

have, however, been found in strawberry essence (Corse and Dimick, 1958).

ACKNOWLEDGMENTS

Financial support of this work was provided by the Western Utilization Branch, ARS, U. S. Department of Agriculture, through a grant to Professor G. Donald Sherman, Hawaii Agricultural Experiment Station.

NMR spectra were determined by Dr. A. A. Bothner-By, Harvard University, and Professor L. Mandell, Emory University.

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Volatile Esters of Bartlett Pears [a, b]

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(Manuscript received June 10, 1961)

SUMMARY

Esters are responsible for the typical desirable aroma of Bartlett pear. Hydrolysis products of these esters were resolved by gas chromatography. The major ester acid is an unsaturated 10 carbon acid, which hydrogenates to yield *n*-capric acid, as identified by relative retention volume and infrared spectroscopy. Nonanoic acid is present in relatively large amounts, and smaller amounts of acetic, propionic, butyric, caproic, and caprylic acids are involved. The major ester alcohols include *n*-butyl alcohol and *n*-hexyl alcohol. Two other still unidentified alcohols, possibly polyfunctional or cyclic, occur in large amounts. The author was unable to find evidence of ethyl esters.

Most fresh fruits are characterized by particular natural aromas that strongly influence buyer appeal and consumer acceptance. These volatile flavor compounds, though the object of much study, remain largely unidentified. Redgrove (1939), reporting on synthetic formulations, implied that a blend of several materials was essential for satisfactory imitation of the pear flavor. His list included, in decreasing order of importance, isoamyl acetate, ethyl acetate, citrus oils, butyl acetate, isobutyl acetate, ethyl butyrate, and isoamyl butyrate.

Mehlitz and Matzik (1956) studied the volatile acids of several pear varieties, not including the Bartlett. They reported volatile acid contents ranging from 44 to 106 mg/L for various varieties, and a volatile ester content of 7.6 mg/L for the Guyot. Formic and acetic acids, identified by paper chromatographic techniques, were found in all varieties; no other acids were reported.

Antoniani *et al.* (1954), Antoniani and Serini (1955), and Serini (1956) demonstrated the presence of 2,3-butylene glycol and acetoin in pears and apples, and suggested that, because the amounts and ratio varied with fruit maturity, these might serve as an index of ripeness. Amoore (1952), attempting to relate certain measurable chemical or physical properties of specific compounds to the odor response generated, predicted that methyl-carbomethoxypimelate would have ethereal, floral, and faint minty components that together would yield a pear aroma. Johnston (1960) submitted this ester to an odor panel under closely controlled conditions, and found that, though flow rate and concentration affected response, at most levels a fruity-pungent odor with floral overtones was obtained.

In a study designed to identify the volatiles of Bartlett pears and evaluate their roles in determining flavor, an enclosed processing system has been utilized that permits recovery of the volatiles in an aqueous essence (Jennings *et al.*, 1961). The work reported here concerns the identity of several of the esters in this essence.

METHODS

Preparation of essences. The essences used were recovered from fresh, ripe Bartlett pears processed in an enclosed system previously described (Jennings *et al.*, 1961).

Preparation of essence hydrolysate. Sufficient C.P. potassium hydroxide was added to aqueous essence to achieve a solution 0.1*M* in hydroxide. This was boiled under reflux for six hours, and cooled.

[a] This project was supported in part by funds from Pear Zone No. 1.

[b] Presented as paper No. 25 at the 21st Annual Meeting, Institute of Food Technologists, New York, 1961.

Preparation of extracts. Aqueous essences, saturated with C.P. sodium chloride, were extracted four times with *ca.* one-tenth their volume of A.R. ethyl ether. The ester alcohols were extracted as described above from the alkaline essence hydrolysate. Ester acids were recovered by acidifying the residue from the alkaline extraction with C.P. hydrochloric acid. The fatty acids liberated by the hydrolysis were then extracted with ethyl ether, as described above. Control extractions indicated that none of the compounds discussed below originated from the ethyl ether.

Preparation of methyl esters. The ether extract of the acidified essence hydrolysate was dried over C.P. sodium sulfate and filtered into a small round-bottom flask, and most of the ether was removed in a rotary flash evaporator. Boron-trifluoride-methanol (Applied Science Laboratories, Inc., State College, Pa.) was added in excess, and the mixture was heated 5 min on a steam bath. The mixture was poured into five times its volume of cold water, and 5 cc isopentane was added to assist in the separation of shorter-chained esters (suggested by R. E. Kepner, Chemistry Department, Univ. of California). The ether layer was separated, dried over C.P. sodium sulfate, and concentrated in a rotary flash evaporator.

Gas chromatography. A commercial gas chromatograph was used, with a four-filament thermal conductivity cell, and helium as the carrier gas. Columns were stainless steel, ¼ in × 10 ft, packed with 20% diethylene glycol succinate (DEGS) on chromosorb. The column was maintained at 175°C and the injector at 250°C, and in those runs where fractions were collected, the collector was maintained at 250°C. Flow rate was 30 cc per minute.

Hydrogenation. Hydrogenation was performed by placing 1–2 μl of the methyl ester mixture in a glass-stoppered 20-cc Erlenmeyer flask. Five ml of isopentane and 3 mg of palladium black catalyst were added. The flask was flushed with hydrogen and placed on a magnetic stirrer. Periodically, fresh hydrogen was flushed through the flask. After 2 hr, the mixture was filtered into a conical test tube, and the major portion of the solvent removed in a rotary flash evaporator.

RESULTS AND DISCUSSION

Fig. 1 illustrates a chromatogram typical of the ether extract of pear essence, and Fig. 2 shows the chromatogram of the ether extract of the alkaline hydrolysate of pear essence. Even under alkaline conditions, the typical pear aroma is quite strong, but when conditions for hydrolysis are made stringent enough that the chromatograms are similar

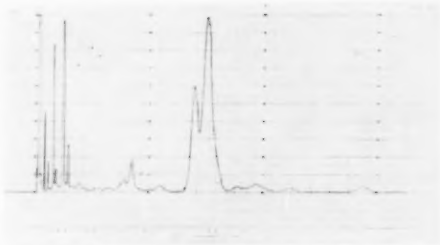

Fig. 1. Chromatogram of the ether extract of pear essence. 175°C, 30 cc/min. Column, ¼ in. × 10 ft, DEGS.

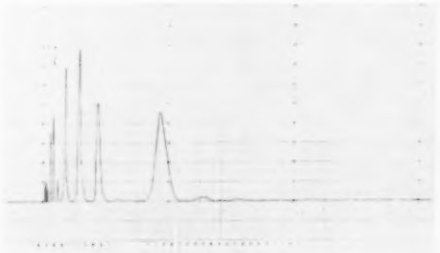

Fig. 2. Chromatogram of the ether extract (alkaline conditions) of the alkaline hydrolysate of pear essence. 175°C, 30 cc/min. Column, ¼ in. × 10 ft, DEGS.

to that in Fig. 2, the desirable pear aroma disappears and other odors become apparent. This indicated that the desirable pear aroma is largely due to volatile esters.

Fig. 3 shows a semilog plot of the retention, relative to that of *n*-hexyl alcohol, of a series of known alcohols as a function of their molecular weights. The arrows at the top indicate the relative retentions of the four major peaks in Fig. 2, which are presumably pear ester alcohols. Note that the first two of these agree with *n*-butyl and *n*-hexyl alcohol. The third and fourth do not agree with any normal straight-chain alcohol. Because an unsaturated compound generally exhibits a slightly greater retention volume on DEGS than does its saturated counterpart (Lipsky *et al.*, 1959), it seemed possible that pear alcohol 3 could be an unsaturated C-7 alcohol. Experimental determination showed that its retention volume on the DEGS substrate agreed precisely with that of 3-heptene-1-ol.

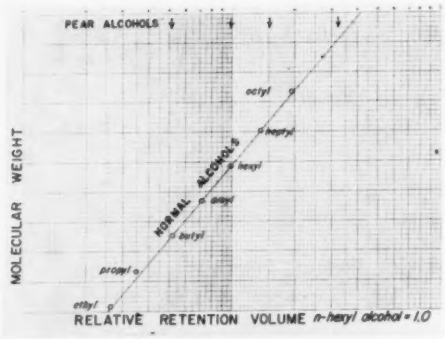

Fig. 3. Comparison of the relative retentions of pear alcohols (top arrows) and a series of known primary *n*-alcohols. *n*-hexyl alcohol = 1.0.

When the pear alcohols were chromatographed on a diglycerol substrate, a material that resolves alcohols to a degree that was previously impossible, pear alcohols 1 and 2 were confirmed as *n*-butyl alcohol and *n*-hexyl alcohol. The relative retention of pear alcohol 3, however, was shown to differ from that of 3-heptene-1-ol on this column. (These chromatograms were run on equipment of Professors R. E. Kepner and A. D. Webb, respectively Departments of Chemistry and Enology, University of California, Davis. A report on the use of this substrate will appear shortly.)

Infrared spectroscopy of pear alcohols 3 and 4 showed similar spectra. The spectra show evidence of hydroxyl groups, carbonyl groups, and methyl groups. One fraction has been isolated from a commercial preparation of acetoin whose retention on DEGS agrees with that of pear alcohol 3. There are differences, which might be reconciled, in their infrared spectra. Their retention values on the diglycerol column have not yet been compared. The possibility of terpene alcohols or other cyclic structures cannot be discounted at this time. Strong infrared absorption at 5.6 μ can be interpreted as indicating a strained double bond in pear alcohol 3. Beyond this, the structure and identity of pear alcohols 3 and 4 remain to be elucidated.

The author was somewhat surprised at the absence of ethyl esters. It was considered possible that the extracting solvent, being in vast excess, was masking the presence of ethyl alcohol. A preparative-scale gas chromatograph was used to check this, by collecting that area of the ether peak where ethyl alcohol would have been—what might be termed an ethanol center-cut. Several such collections were combined, rechromatographed, and then retrapped at the point where ethanol would have emerged. This material was then examined on ¼-in. analytical columns containing DEGS, and on another of diglycerol, whose retention time permits ethanol to separate further from ether. Ethyl alcohol was not demonstrated. Thus, if any ethyl esters do exist in pear, they appear to be present in relatively insignificant amounts.

The ester acids were isolated by acidifying the alkaline hydrolysate and extracting with ethyl ether. Fig. 4 shows a chromatogram of the methyl esters of pear ester acids on DEGS at 175° and a flow rate of 40 cc/min. Fig. 5 shows a hydrogenated sample under the same conditions. Note that two major changes are occasioned by hydrogenation. The major peak in Fig. 4, which falls between the positions that would be occupied by the methyl esters of the normal C-11 and C-12 acids, is shifted to agree with methyl caprate. There were several other changes, somewhat less striking, such as the disappearance of three other peaks and the appearance of another on hydrogenation.

On two previous occasions, hydrogenation

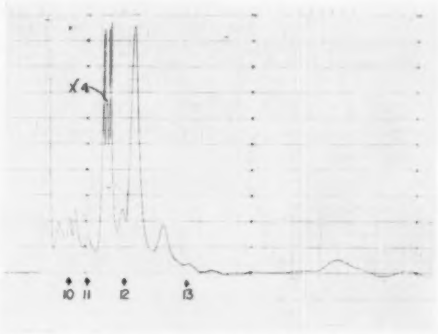

Fig. 4. Chromatogram of methylated pear ester acids. 175°C, 40 cc/min. Column, ¼ in. × 10 ft, DEGS.

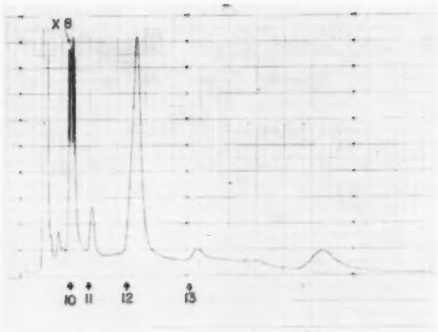

Fig. 5. Chromatogram of methylated hydrogenated pear ester acids. 175°C, 40 cc/min. Column, ¼ in. × 10 ft, DEGS.

resulted in a decrease of this major peak, and the appearance of a new peak with a much longer relative retention. Together with absorptions in the infrared spectra that could be explained on the basis of more than one nonequivalent carbonyl group, this was at first interpreted as evidence of keto acids that hydrogenated to the hydroxy form. On this column, such a conversion would cause a longer retention time (Lipsky *et al.*, 1959). Later work indicates that these results were artifacts caused by experimental procedure. These earlier methylations were done by refluxing over concentrated sulfuric acid; in the current work, the relatively new BF_3 reagent was used, resulting in methylation with much milder treatment. Even when samples were drawn out before hydrogenation had proceeded to any great extent, evidence for production of the slower component no longer exists.

Fig. 6 shows a chromatogram of the free, unmethylated pear ester acids on Empol acid dimer column at 198°C, and a flow rate of 87 ml He/min. A report on the use of this substrate (suggested by Drs. Webb and Kepner) will appear shortly in *Analytical Chemistry*. Fig. 7 shows a sample of hydrogenated pear ester acids chromatographed under the same conditions. The numbers and arrows at the very bottom of each figure represent the positions occupied by the appropriate straight-chain *n*-fatty acids. Study of Fig. 6 suggests that pear esters contain some acetic acid, a trace of propionic acid, evidenced as a shoulder, a small amount of butyric acid, one that might be isovaleric acid, caproic acid, and caprylic acid. There is a major component that agrees with nonanoic acid, one agreeing with capric acid, and a very large one that is in the neighborhood of, but doesn't coincide with, lauric acid. Though hydrogenation exerts little influence on the first portion of this curve, it has a profound effect on the major component, which shifts and now occupies a position corresponding to that assigned to *n*-capric acid.

Fig. 8 shows, in the upper half, the infrared spectrum of the major pear ester acid, unhydrogenated, and in the bottom curve, the same material after hydrogenation. This bottom curve agrees precisely with the infrared spectrum of *n*-capric acid. The major differences between these two spectra, e.g., the disappearance of absorption bands at 6.2, 7.1, 10.1, and 11.5 μ, can all be accounted for on the basis of hydrogenation of methylene groups.

On the basis of relative retention times of the pear acids and their methyl esters on

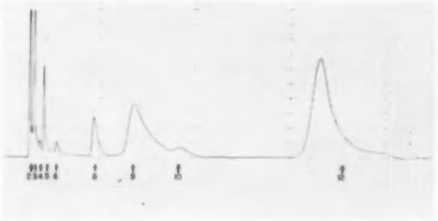

Fig. 6. Chromatogram of pear ester acids. 198°C, 87 cc/min. Column, ¼ in. × 10 ft. Empol dimer acid.

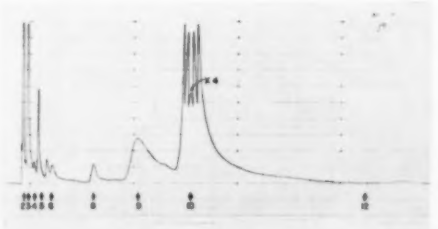

Fig. 7. Chromatogram of hydrogenated pear ester acids. 198°C, 87 cc/min. Column, ¼ in. × 10 ft. Empol dimer acid.

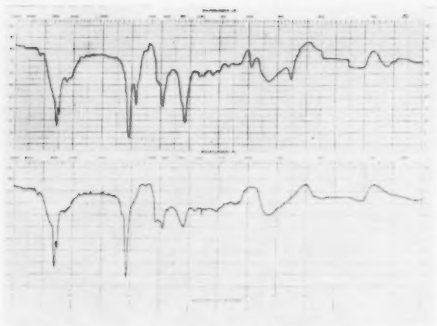

Fig. 8. Comparison of the infrared spectra of the major pear ester acid before (top curve) and after (bottom curve) hydrogenation. Approx. 2% solutions in CCl_4, micro-cavity cell.

these two columns, it seems reasonable to conclude that small quantities of acetic, butyric, caproic, caprylic, and capric acids are involved in these esters. There is a trace of propionic acid, and possibly some isovaleric acid. Nonanoic acid is present in larger amounts, and the major pear acid is an unsaturated C-10 acid.

We are currently engaged in preparative-scale gas chromatography to isolate larger quantities of pear esters and investigate which acids are normally esterified with which alcohols.

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Volatile Flavor of Sauerkraut.[a, b] Gas Chromatographic Identification of a Volatile Acidic Off-Odor

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(Manuscript received June 19, 1961)

SUMMARY

A method is presented for gas chromatographic identification of the chain length and structure of methyl esters of lower-molecular-weight fatty acids. The chemical identity of the "cheese-like" off-odor of sauerkraut was established. Abnormally high concentrations of *n*-propionic, *n*-butyric, and *n*-caproic acids were found in samples of kraut that had been graded low because of this off-odor defect. A very poor sample that had been discarded, contained isobutyric and isovaleric acids in addition to *n*-propionic, *n*-butyric, *n*-valeric, and *n*-caproic acids. In this sample, *n*-butyric acid was found in the largest concentration—103 ppm.

INTRODUCTION

The primary reaction occurring during the fermentation of cabbage to sauerkraut by the lactic acid bacteria is the conversion of carbohydrates to acids and alcohol. The identification of acetic and lactic acids and ethyl alcohol as the major end products of fermentation has been well-established (Pederson, 1960). At times, abnormal fermentations occur, producing sauerkraut with various types of off-odors. An off-odor that is observed on occasion has been described as "cheese-like" to putrid. Although relatively rare today, this off-odor still presents a problem of serious economic loss to the industry.

In attempting to establish the chemical nature of this off-odor, it was initially postulated to be associated with the breakdown of the sulfur-containing constituents of the cabbage, particularly the thioglucosides and sulfur-containing amino acids. Many of the unpleasant odors produced during the cooking of some vegetables have been associated with the breakdown of the various labile sulfur compounds. The possible role of hydrogen sulfide or volatile mercaptans was eliminated by the use of a "chemical odor filter" (Brenner *et al.*, 1955). The addition of a 5% copper sulfate solution, which completely screens out the odor of both hydrogen sulfide and mercaptans, produced no significant change in the "cheese-like" aroma of the off-odor kraut. However, neutralization of the steam distillate removed the off-odor, suggesting that lower-molecular-weight fatty acids, other than acetic, were responsible for the off-odor defect.

EXPERIMENTAL

The material studied was samples of canned sauerkraut graded according to U.S.D.A. standards for canned sauerkraut ("United States Standard for Grades of Canned Sauerkraut." United States Department of Agriculture, Agricultural Marketing Service, Washington. D.C., May 10, 1957). The off-odor samples consisted of one sample graded substandard and discarded because of the off-odor defect, and four others showing varying degrees of the off-odor. Controls were two samples graded excellent.

Six hundred grams of sauerkraut were steam distilled at the rate of 5 ml per min. A Friedrich condenser and ice bath served to collect the distillate. Approx. 1200 ml of distillate were collected. The distillates were adjusted to pH 8.3 with 0.1*N* NaOH and concentrated in a rotary film evaporator. Following acidification of the sodium salts, the free

[a] Approved for publication by the Director of the New York State Agricultural Experiment Station as Journal Paper No. 1212.

[b] Presented in part at the 21st annual meeting of the Institute of Food Technologists, New York, May, 1961.

acids were recovered by extraction with ethyl ether. The comparatively large excess of acetic acid, which is normally present in sauerkraut, was removed by silicic acid chromatography by the method of Marvel and Rands (1950) as modified by Rice and Pederson (1954). The effluent-volume pattern showed that all of the lower-molecular-weight fatty acids, except for acetic and formic, were found in the first eight fractions. After titration with 0.01*N* methanolic NaOH, these fractions were combined and evaporated to dryness. The acids were liberated with 10% H_2SO_4 and extracted with a minimum of ethyl ether. The efficiency of recovery of the acids from the silicic acid column was 97%. The ether extract was concentrated in a stream of nitrogen at 4°C. The fatty acids were converted to the corresponding methyl esters by reaction with diazomethane (Vorbeck *et al.*, 1961).

A Barber-Colman Model 10 gas chromatograph, employing an ionization detection cell containing 56 microcuries of Radium 226, was used for qualitative and quantitative determination of the methyl esters of the fatty acids. Since retention data on a single stationary phase can be misleading, two chemically distinct types of stationary phases were used to define the chain length and structure of the unknown acids.

The U-shaped columns were of heavy-walled boro-silicate glass tubing, 5 mm ID and 6 ft long. The stationary phases used were Apiezon L, a non-polar saturated paraffin hydrocarbon, and 1,4-butanediol succinate (BDS), a polar polyester of succinic acid. The Apiezon L was packed on Chromasorb W, 80–100-mesh, in a ratio of 1:5 (w/w). The BDS was packed on Chromasorb P, 60–80-mesh, in a ratio of 1:5 (w/w) (Vorbeck *et al.*, 1960). Both columns were preconditioned by baking prior to analytical use (90°C for the Apiezon L and 85°C for the BDS). The operating parameters were: Apiezon L column: temperature 84°C, cell 235°C, flash heater 206°C, argon pressure 30 psi, flow rate 79 ml per min. BDS column: temperature 79°C, cell 245°C, flash heater 216°C, argon pressure 17 psi, flow rate 33 ml per min. A potential of 500 volts was applied to both detectors, and argon was used as the carrier gas for both columns.

A mixture of known composition containing both normal and branched-chain saturated acids was prepared and chromatographed to obtain relative retention data. Fig. 1 shows the relationship obtained by plotting the log of the relative retention volume in a polar and nonpolar stationary phase. The retention volumes were calculated relative to methyl butyrate. The slope of the line is dependent on the relative interactions of the esters with the two stationary phases. Differences in London dispersion interactions with the stationary phase control the separation of the normal and branched-chain saturated esters (James, 1959). In the polar BDS phase, the retention volumes are smaller than in the nonpolar Apiezon L because the London dispersion interactions between the esters and the polar phase are smaller in magnitude. With this technique, 2-methyl butyric, 3-methyl pentanoic, and 2-methyl pentanoic acids can be separated and identified (unpublished data).

RESULTS AND DISCUSSION

Fig. 2 shows a tracing of the chromatograms of the esters from the lower-molecular-weight fatty acids of the off-odor kraut and excellent kraut. A considerable difference is observed between the two samples in both the nature and amounts of the lower-molecular-weight fatty acids present. The relative retention volumes in both Apiezon L and BDS were calculated, and the peaks are identified from the data presented in Fig. 1.

Table 1 shows the distribution of the lower-molecular-weight fatty acids in the two samples of sauerkraut. The amounts of the various acids were calculated by triangulation from the peak areas. Previous work from this laboratory (Vorbeck *et al.*, 1961)

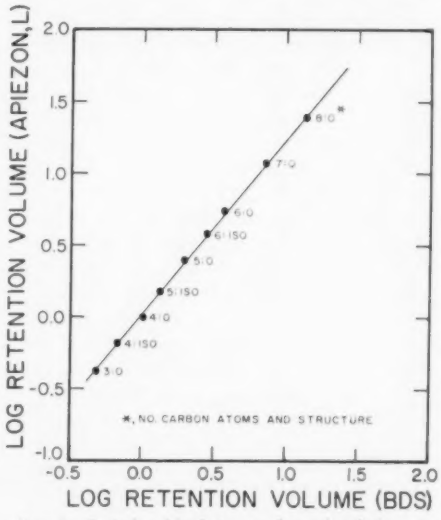

Fig. 1. Relationship between log of relative retention volumes of normal and branched-chain saturated acids in a polar (BDS) and nonpolar (Apiezon, L) substrate. Retention volumes calculated relative to methyl butyrate.

Table 1. Distribution of lower-molecular-weight fatty acids.

Acid	Concentration (ppm)	
	Excellent kraut (EK)	Off-odor kraut (PK)
Propionic	0.1	6.7
n-Butyric	0.1	103.0
iso-Butyric	—	3.9
n-Valeric	—	19.2
iso-Valeric	—	9.9
n-Caproic	1.4	59.3
n-Heptanoic	10.9	—
n-Caprylic	10.0	7.1

has shown a molar-area relationship for the ionization detector used. The standard deviation of the method is $\pm$ 0.32%.

The most striking difference is in the concentration of *n*-butyric acid. The off-odor kraut contained 103 ppm, approx 1000 times as much *n*-butyric acid as in the excellent kraut. Isobutyric, isovaleric, valeric, and *n*-caproic acids were also found in significant concentrations in the off-odor kraut, but were notably absent or insignificant in the excellent kraut. The presence of heptanoic acid in the excellent kraut is interesting.

n-Butyric, *n*-valeric, and *n*-caproic acids added to the excellent kraut in the concentrations found in the off-odor kraut produced a similar "cheese-like" off-odor defect. A flavor panel could detect a concentration of *n*-butyric acid of about 7 ppm in an aqueous solution. The effect of degree of ionization of the butyric acid on flavor intensity is currently receiving investigation.

Four other samples of sauerkraut, with less pronounced off-flavor but scored low because of the "cheese-like" odor, were found to contain *n*-butyric acid as well as other lower-molecular-weight fatty acids. The concentrations of the acids were significantly higher in these samples than in the second control sample (Table 2). Three of these (19, 45, 287), like the sample previously dis-

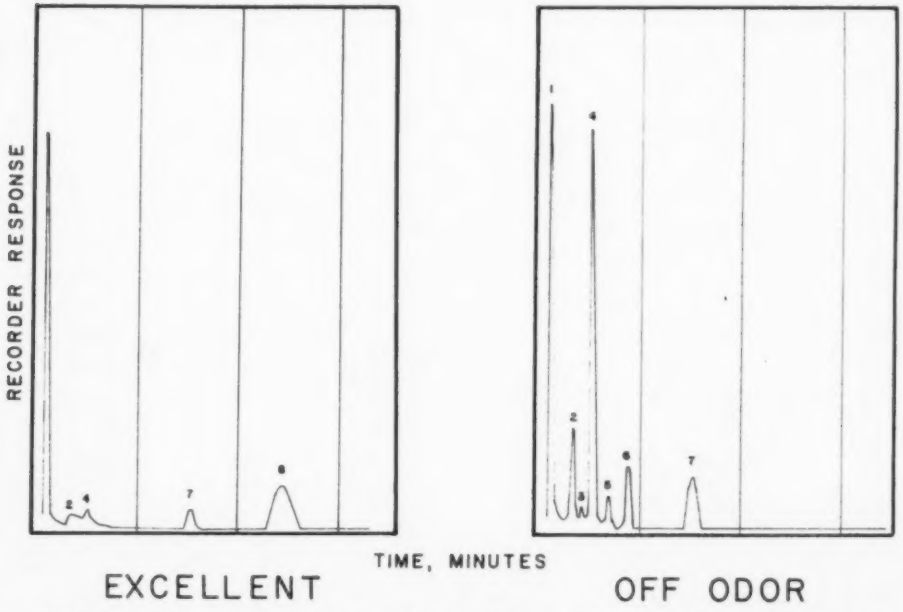

Fig. 2. Chromatograms of methyl esters of lower-molecular-weight fatty acids in off-odor and excellent kraut. BDS stationary phase. 1) solvent (ethyl ether); 2) methyl propionate; 3) methyl isobutyrate; 4) methyl butyrate; 5) methyl iso-valerate; 6) methyl valerate; 7) methyl caproate; 8) methyl heptanoate.

Table 2. Analysis of sauerkrauts graded low because of "cheese-like" off-flavor.

	Control	19	45	287	217
Lower-molecular-wt. fatty acids	Concentration (ppm)				
Propionic	—	72.7	86.7	20.1	14.2
n-Butyric	0.3	42.6	27.1	30.9	37.6
n-Caproic	—	—	4.1	1.7	8.1
n-Heptanoic	7.6	1.5	—	—	0.9
n-Caprylic	3.9	3.8	0.3	2.1	1.2
Grading					
Score	95	89	85	91	83
Grade	A	C	C	B	C
Total Acid	1.42	1.29	1.39	1.27	1.26
Salt	1.60	1.70	1.64	1.63	2.26
Salt/Acid Ratio	1.06	1.32	1.18	1.32	1.88
Ascorbic Acid	18.7	25.1	1.5	7.1	14.5
Color					
Rd	29.0	32.4	27.4	27.0	29.3
a	0.0	−1.1	−0.3	−1.0	+2.0

cussed, had been graded high in all respects except flavor. The objectionable odor and flavor were not so pronounced as in sample PK, and the samples otherwise showed normal analyses for total acid, salt, ascorbic acid, and color.

The mechanism by which the lower-molecular-weight fatty acids were formed is not explained. The large concentrations of these acids in the off-odor krauts appear not to have resulted from simple hydrolysis of the lipid material of the cabbage. A study was made of the fatty acids in the extracted crude lipid of the samples. No detectable amounts of lower-molecular-weight fatty acids were found in the phospholipid or neutral fat of any samples. Whether these acids were the result of a breakdown or synthesis during an abnormal fermentation is not known. It is known that the lower-molecular-weight fatty acids are responsible for the hydrolytic rancid flavor of butterfat and are of importance in the flavor development of many types of cheeses.

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An Investigation of Varietal Differences in the Iodine-Binding Capacities of Crystalline Rice Amyloses [a]

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(Manuscript received May 16, 1961)

SUMMARY

Purified amyloses have been obtained from four widely different varieties of rice by alcohol fractionation of the corresponding starch. Each variety of amylose was found to bind 18.9 mg of iodine per 100 mg of amylose on the basis of solvent-free carbohydrate. Viscosity measurements in potassium hydroxide solutions have made possible the calculation of the viscosity-average molecular weights. These molecular weights were found to be: Century Patna 231, 100,000; Caloro, 140,000; Rexoro, 325,000; and Zenith, 310,000. Beta-amylase action on Century Patna and Rexoro amyloses indicated that the structures of both were similar.

From previous investigations (Rao *et al.*, 1952; Williams *et al.*, 1958), rice varieties may now be characterized by the amylose content of their starches as well as size and shape of the whole grain. Little information is available, however, as to whether any differences exist in the amylose molecules themselves. The molecular weights of several amyloses extracted from rice have been determined previously in this laboratory by a periodate oxidation method (Tsai *et al.*, 1960). Although the amyloses prepared for that study were not completely purified, it was nevertheless possible to calculate minimal molecular weights on the assumption that all varieties would bind the same amount of iodine by weight when completely purified. The present study was undertaken to appraise the validity of that assumption and to provide additional information on the physicochemical nature of rice amyloses.

EXPERIMENTAL METHODS

Isolation and purification of the amyloses. In obtaining the four varieties of rice chosen for this study, no attempt was made to secure uniform nursery samples. Caloro rice was obtained from Biggs Station, California; the Rexoro, Zenith, and Century Patna 231 were obtained from Crowley, Louisiana. All were 1958 crop.

The procedure for extraction of starch, defatting, and subsequent fractionation and purification has been described (Tsai *et al.*, 1960). Purification was extended, however, by repeated washings with butanol-saturated water and recrystallization from hot water as the butanol-amylose complex by the addition of excess butanol. All of the final products were recrystallized five times and washed fifteen times.

The amylose was dried by stirring a suspension of amylose vigorously in butyl alcohol for 1 hr. The butanol was decanted and the treatment repeated three or more times until the product was sufficiently powdered. The product was collected on a suction filter and dried overnight in a vacuum desiccator over concentrated sulfuric acid. The final products were stored in a desiccator over phosphorus pentoxide.

Moisture and ash analyses were made on representative samples of all varieties, including the "standard sample." Moisture content was determined from the weight loss upon drying 18 hr at 80°C in a vacuum oven. Samples were ashed for 2 hr at 500°C in a Lindberg electric furnace.

Iodine affinity. Two methods were employed for determining total iodine bound. The first was a modification of a colorimetric procedure developed in this laboratory (Williams *et al.*, 1958). The amylose was weighed into a volumetric flask and covered with 1*N* potassium hydroxide (usually 5 ml in a 50-ml flask). This solution was allowed to stand 2–4 hr at 4°C. Longer solution time per-

[a] Presented at the 21st Annual Meeting of the Institute of Food Technologists, New York.

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mitted dissolving a greater quantity of amylose, but invariably led to a decrease in iodine binding, presumably through oxidative degradation. After solution was complete, the flask was diluted to volume with distilled water, and an aliquot containing 1.0–1.6 mg of amylose was removed to a 250-ml beaker. To this was added 50 ml of distilled water and the pH was adjusted to a predetermined arbitrary pH meter reading (10.8 in most cases) with 0.06*N* hydrochloric acid. A meter reading of 10.8 corresponded to an indicator paper reading of pH 6–7. This solution was then transferred to a 100-ml volumetric flask, 2 ml of an iodine reagent containing 0.2% iodine in 2.0% potassium iodide was added, and the flask contents were diluted to volume. The color was read in a Klett-Summerson photometer with the K59 filter. The solution color and reading varied with the pH of the solution, but by adjusting to a meter value of 10.8, even a solution containing as little as 1.0 mg of amylose was always a deep blue. The relation of concentration to optical density was linear in the range employed (1.0–1.8 mg amylose).

The second method investigated to provide iodine sorption data was a potentiometric titration. Two procedures were followed: the classical titration method for starch (Bates *et al.*, 1943), and an unpublished method circulated by Dr. T. J. Schoch (1960). This latter method differed from the former in that dispersion of the amylose was carried out for 15 min in concentrated calcium chloride solution (40° Baume) in a boiling water bath. This avoids the possibility of degradation due to overexposure to strong alkali.

The iodine titrant solution for use in either method was prepared fresh daily by a tenfold dilution of 10 ml of a stock solution containing 2.000 g of iodine and 83 g of potassium iodide per liter as determined by standardization against arsenic trioxide and sodium thiosulfate. The concentration of the stock solution did not change appreciably over a 2-month period when stored in the dark. Titrations were performed on 10-mg samples with a 10-ml microburet. A Beckman Model G pH meter was used in conjunction with bright platinum and saturated calomel electrodes for determination of the potential.

Total carbohydrate analyses. The anthrone method (Loewus, 1952) was used for determination of total carbohydrate, using as standard a sample of Rexoro rice amylose prepared by J. T. Hogan (1961), by a slightly different procedure (Wilson *et al.*, 1943).

Viscosity studies. Viscosities of each variety of amylose prepared in this laboratory were measured in 1*N* potassium hydroxide solutions not over 4 hr old. The viscometer was an Ostwald-Fenske pipette, No. 100, with a flow time of approx 60 sec for solvent. Flow times were measured to the nearest 0.01 second with a stopwatch; the temperature was controlled with a circulating water bath thermostated at 30.0°C. For each determination, the alkaline solution was allowed to warm to room temperature, then was diluted to volume with additional potassium hydroxide solution, and filtered through a coarse sintered-glass funnel. The pipette was filled with 10.0 ml of solution and allowed to equilibrate 10 min in the bath. Four or five readings were made per filling.

Enzymatic analyses. The Rexoro and Century Patna 231 varieties were subjected to the saccharogenic action of crystalline beta-amylase. The enzyme was obtained from Nutritional Biochemicals Corp. The reaction was conducted for 24 hr at 30°C in 0.2*M* acetate buffer, pH 4.8. Reducing sugars formed were determined by the dinitrosalicylic acid method (Sumner, 1925), using maltose as the standard.

RESULTS AND DISCUSSION

To obtain reliable information on the iodine-binding capacity (IBC) of each amylose, complete purification was necessary. When purification had been extended until the iodine sorption became constant, the amylose was assumed pure. On this basis, the pure amyloses from the four varieties were found to bind essentially the same amount of iodine by weight. However, when compared to the "standard sample" of Rexoro rice amylose suplied by J. T. Hogan, the amylose samples were found to be only 83% amylose by both colorimetric IBC and carbohydrate analyses (Table 1). For further confirmation, potentiometric titrations were then conducted on each variety and the standard. Fig. 1 shows the results of two potentiometric titrations; the Zenith amylose shows only 15.7 mg for IBC, compared to 18.8 mg for the standard sample. By this

Table 1. Carbohydrate and blue values.

Variety	% carb.	Blue value [a] (590 mμ)
Century Patna 231	83.0	0.306
Rexoro	82.5	0.312
Caloro	84.0	0.316
Zenith	82.0	0.309
"Standard"	100.0[b]	0.366

[a] Optical density corresponding to a concentration of 1 mg of amylose per 100 ml final volume.
[b] Assumed.

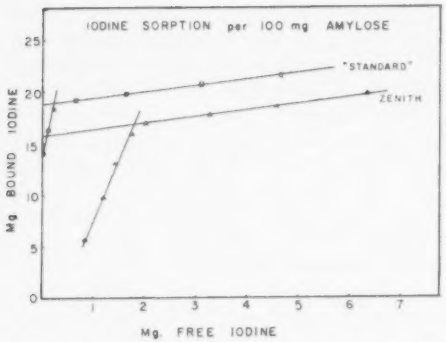

Fig. 1. Potentiometric iodine titration of Zenith and "standard" amylose, both corrected to dry weight but not corrected for total carbohydrate content.

method, along with the carbohydrate content analyses, it was possible to determine that the IBC of all the amyloses prepared in this laboratory was 18.9 mg iodine per 100 mg of amylose. Japanese workers (Inoue and Onodera, 1952) reported that the intensity of the iodine coloration with amylose varies with the variety of rice. The results with these four American varieties do not indicate this, but instead that any variations reported in IBC must be due to differences in the method of preparation and purity of amylose rather than any varietal differences.

The nature of the non-carbohydrate contaminant was also investigated. Because protein, lipid, and ash content of the amyloses was negligible, and because moisture content of all the amylose varieties had been determined, the impurity present in the amylose is felt to be solvent bound into the crystal. Carbon and hydrogen analysis of the Century Patna amylose by an independent laboratory (Weiler and Strauss, 1961) revealed the presence of approx 10% bound water when calculated on the basis of an analysis of 40.26% C and 6.34% H. These analytical data cannot be explained if either butanol or any combination of butanol and water is the contaminating agent; only water is indicated present. The failure of the analysis to account for all 17% of the contaminant must be due to the predrying (temperature and time unavailable) before the analysis. All attempts made in this laboratory to remove the contaminating material by moderate heating (less than 100°C) or vacuum sublimination (0.05 mm pressure) were unsuccessful. On heating 18 hr *in vacuo* at 100°C (with 18 hr *in vacuo* at 80°C as base), weight loss was 2%; heating 16 hr at 175°C, weight loss was 10%. Thus this contaminating water cannot be merely present as moisture in the sample (true moisture content varied from 3–5% in the samples and was taken into consideration in all instances).

Since the incorporation of complexed water into the amylose crystals may have resulted from rapid crystallization, an extension of the time of crystallization might avoid incorporation of large amounts of solvent and obviate the need for carbohydrate analyses. All of the recrystallizations of the "standard" amylose were carried out slowly in insulated flasks (Hogan, 1961) and were free of bound solvent. This point is being investigated.

Other viscosity studies (Everett and Foster, 1959) have made it possible to determine the viscosity-average molecular weights for the amyloses examined here. The investigation cited provided some idea of the conformation of amylose in solution, thereby furnishing data that might be used under similar conditions to estimate the molecular weights of any amylose.

Intrinsic viscosities were obtained with Wagner's equation (Wagner, 1947):

$$/\eta/ = \frac{\log_e \eta_{rel}}{c} + k\left[\frac{\log_e \eta_{rel}}{c}\right]^2$$

where c = concentration in g/deciliter
and k = empirically determined constant

Preliminary investigations of the applicability of this equation revealed that it could be used with high accuracy in the concentration range of 0.1–0.4 g/deciliter. For a concentration of 0.2%, k was found to be 0.038. This is in good agreement with the value of k determined previously for amylose in potassium hydroxide (Wolff *et al.*, 1950).

The intrinsic viscosity calculations from relative viscosity data at 0.2% concentration are given in Table 2. These represent the average of two separate determinations at the same concentration. All determinations on the same samples were within 2% of one

Table 2. Molecular constants.

Variety	Int. visc.	Mn [a]	Mv
Century Patna 231	0.84	29,000	100,000
Caloro	1.10	52,000	140,000
Rexoro	2.09	41,000	325,000
Zenith	2.00	41,000	310,000

[a] From Tsai *et al.*, 1960.

another. All weights of samples were corrected for moisture and other non-carbohydrate content.

The viscosity–average molecular weights of the amyloses were calculated from the modified Staudinger equation, using the constants evaluated by Everett and Foster:

$$/\eta/ = K'M^a$$

where $K' = 0.85 \times 10^{-1}$ deciliters/g

$a = 0.76$

$M =$ molecular weight $\times 10^{-5}$

In addition to the viscosity-average molecular weights (Mv), Table 2 also includes molecular weights (Mn) obtained previously (Tsai *et al.*, 1960) using periodate oxidation techniques. It has been shown (Schaefgen and Flory, 1948) that for linear polymers having a most probable distribution, Mv/Mn would be approx 1.85 for *a* equal to 0.76. In every case for our amyloses, the ratio of Mv/Mn is greater than this value, indicating a predominance of larger molecules. This provides good evidence that relatively little or no degradation has taken place as a result of the preparative procedure or the presence of alkali. It might also indicate that the numerous recrystallizations produced a subfractionation of the amylose, and that the smaller molecular species were absent. If that be the case, it should have occurred to nearly the same extent in all samples, and the comparisons are still valid.

The values of the intrinsic viscosities (and the viscosity molecular weights) appear to be sufficiently different for the different amylose varieties so as to reflect true variations in molecular size. No positive statement can be made until the extent of variation among several samples of the same variety can be determined. Whereas the number-average molecular weights gave a minimum value for the molecular weight and showed little indication of varietal differences, the "true" molecular weight (as expressed by a weighted value such as the viscosity-average molecular weight) points up a much greater difference in size. Before attempting to correlate other varietal studies with the molecular weights as presented here, it will be necessary to obtain some knowledge of differences in the molecular weights of the amylopectins.

The action of beta-amylase was studied on the Rexoro and Century Patna 231 amyloses. Since beta-amylase is apparently able to degrade completely only glucose polysacharides containing uninterrupted alpha-1,4 linkages, this method could be used to show their similar chemical nature even though these varieties exhibited the greatest difference in intrinsic viscosity. With a commercial thrice-crystallized enzyme preparation reported free of alpha-amylase activity, these amyloses were respectively converted 96% and 99% to maltose. These values for the beta-amylase limits imply that the amyloses are identical in structure and possess an essentially linear arrangement.

FOOTNOTE TO THE STUDY

Since submitting this paper the authors have obtained a re-analysis of the Century Patna amylose referred to above as containing 10% complexed water at the time of the initial analysis. After 3 mo. of storage over P_2O_5 the sample was found to be 44.5% C and 6.17% H, compared to the theoretical values of 44.4% C and 6.23% H for anhydrous amylose. Not only was it free of solvent, but it was also partially retrograded as evidenced by the slight turbidity produced on dissolving it in 1*N* KOH. These findings suggest that the solvent that could not be removed by moderate heating was gradually released as the amylose aged over P_2O_5.

ACKNOWLEDGMENT

The authors express their appreciation to the Institute of Food Technologists and the General Foods Corporation for generous financial assistance in the form of a General Foods Fellowship awarded one of the authors.

Sincere thanks are also extended to Mr. J. T. Hogan for assistance and comments.

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Volatile Organic Acids in Pre-ferments for Bread

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(Manuscript received July 20, 1961)

SUMMARY

An ionization-detector gas chromatographic unit was used to analyze the acids extracted from a pre-ferment typical of those used in commercial bread-making. At least 45 acids were separated as esters on a column containing an adipate polyester of diethylene glycol. Seventeen of the monocarboxylic acids were identified by relative retention times in free acid form. Their identities were confirmed by preparing and separating their esters directly by flash exchange gas chromatography. The acids thus identified are: formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, crotonic, isocaproic, caproic, heptylic, caprylic, pelargonic, capric, lauric, myristic, and palmitic.

The recent trend in the baking industry toward replacing sponge fermentation in bread-making by procedures using pre-ferments has created an increased demand for knowledge of flavor substances produced during fermentation. It is well established that the main end products of panary fermentation are ethyl alcohol and carbon dioxide; however, many other compounds are formed that have definite flavor values. For a complete flavor picture, these must be determined. Of the many compounds released into the surrounding media by yeast cells during fermentation, organic acids can be separated most easily. This, coupled with the observation made in our laboratory that a mixture of organic acids isolated from a typical pre-ferment gave rise on heating to a strong aroma reminiscent of freshly baked bread, prompted an investigation into the organic acids of bread pre-ferment.

Among earlier investigations on the organic acids produced during bread-making, Baker *et al.* (1953), using colorimetric and titration techniques, found acetic acid. This acid was present in very small amounts and was felt by them to have little effect on flavor and aroma. Johnson *et al.* (1958) used paper and column chromatography to separate acids and derivative formation, as well as infrared spectra to identify acetic and lactic acids in pre-ferments. A third acid found by them was not identified.

With the advent of gas chromatography, the number of acids found increased somewhat. Wiseblatt (1960) identified acetic, *n*-butyric, iso-valeric, and *n*-caproic acids in fermented dough, bread extracts, and oven vapors.

The present work describes identification of seventeen organic acids in pre-ferments typical of those used in commercial bread production.

MATERIALS AND METHODS

The gas chromatographic unit used was a Barber-Colman Model 10 with an ionization-detector cell containing 20 millicuries of strontium-90. The cell was operated at 220°C and a potential of 1100 V. The U-shaped column, heavy-walled Pyrex glass tubing, 4 mm ID and 8 ft long, was packed with LAC 1-R 296 (glycoladipate polymer) (5 parts) on acid-washed fire-brick (40-60 mesh) (95 parts). The column temperature was varied to accommodate the type of material being analyzed. A preheater maintained at 275°C surrounded the inlet to the column. Gas (argon) flow rate was 100 ml/min, with an inlet pressure of 10 psig and atmospheric outlet pressure. Except in Fig. 1, all

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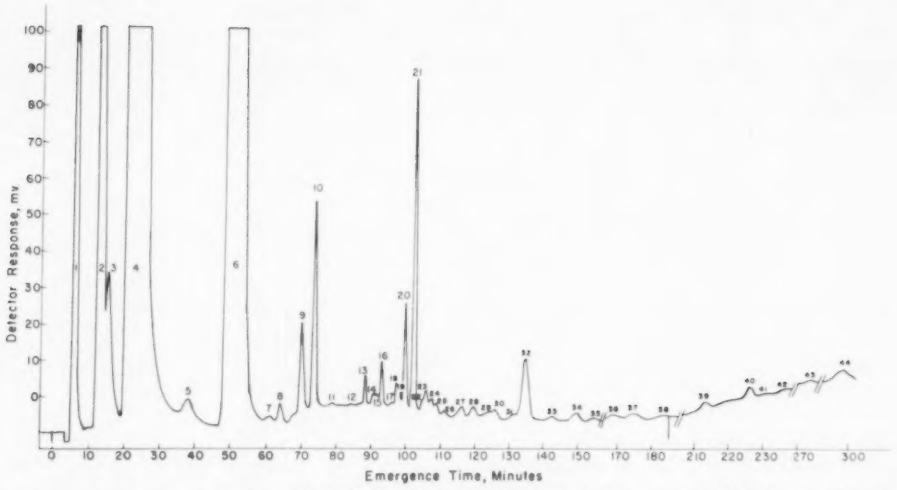

Fig. 1. Programmed heating (35–130°C) of the ethyl esters of the organic acids of a bread pre-ferment prepared by flash exchange gas chromatography.

data reported here were obtained with a fixed column temperature.

Fermentation liquor. A pre-ferment such as used in certain methods of bread production was prepared by incubating the following mixture aerobically for 6 hr at 37°C while stirring, and centrifuging to obtain the clear, cell-free liquor:

	Grams
Glucose	330
Salt	110
Brew improver [a]	33
Compressed yeast	220
Water	5000

Organic acids. The carboxylic acids used for comparative purposes were commercial samples. All yielded single peaks when tested individually in the gas chromatographic apparatus. These acids were used in making up synthetic acid mixtures and also in preparing standard solutions of sodium or potassium salts to be used in making synthetic salt mixtures as described in an earlier publication (Hunter, 1961).

Potassium ethyl sulfate. Prepared following the direction of Evans and Albertson (1917).

The organic acids were extracted from the described cell-free pre-ferment with methylene chloride and concentrated as sodium salts essentially as described by Hunter *et al.* (1960) except that the last 100 ml of solution were concentrated to dryness in a tray lyophilizer under vacuum (0.05 mm Hg) with the trays at 33°C. The organic acids were regenerated with dichloroacetic acid and separated by gas chromatography as described earlier (Hunter *et al.*, 1960). The main features of the flash exchange procedure used for the preparation and separation of the ethyl esters of the organic acids have also been described (Hunter, 1961). However, the following slight modifications were introduced for these analyses. Twenty-two-gauge three-inch-long hypodermic needles were used as well as two-inch-long needles. The contents of the filled needles were dried 10 min under vacuum (500 mm Hg) at 100°C.

Separations of unknown and synthetic mixtures of the acids or esters were carried out isothermally at several temperatures. Appropriate compounds were selected at each temperature for determination of the retention times relative to these compounds.

[a] A mixture of salts having the following composition: ammonium monohydrogen phosphate 200 g, potassium sulfate 50 g, magnesium sulfate 25 g, and calcium carbonate 50 g.

RESULTS AND DISCUSSION

The relative retention times shown in Table 1 provide tentative identification of 17 monocarboxylic acids in a pre-ferment. Identity of the acids was confirmed by flash exchange preparation and chromatographic separation of the ethyl esters of pre-ferment acids and of known acids. Relative retention times of the esters are shown in Table 2.

Programmed flash exchange chromatography of the organic acids in an extract of a pre-ferment revealed at least 44 components (Fig. 1). These include mono- and probably di- as well as other types of carboxylic acids, the dicarboxylic acids appearing both as mono- and di-ester.

The appearance of the higher-boiling fatty acids among the free acids leads to speculation that they may play an unsuspected role in bread flavor and aroma. They could conceivably hinder the volatility of lower-boiling components and increase the time required for their evaporation out of bread. Fairly generous amounts of the acids between isobutyric and caproic were observed by Hunter *et al.* (1960). However, it is interesting to note that in aggregate these acids give rise to the delightful aroma that is usually associated with baking bread.

The odor of the mixture of acids extracted from pre-ferments is pleasant, though not particularly bread-like until the mixture is heated. Upon warming over an open flame the acid mixture gives off a persistent and pleasant aroma quite reminiscent of bread. A similar faint odor is always noted when aliquots of the free acids from a pre-ferment are injected into the heated entry port of the chromatographic column.

Table 1. Relative retention times of aliphatic carboxylic acids from a bread pre-ferment and synthetic acid mixture.

Acid	Relative retention time: Synthetic mixture	Relative retention time: Pre-ferment	Column temperature (°C)
Formic	0.16	0.17	120
Acetic	0.28	0.26	
Propionic	0.40	0.40	
Isobutyric	0.49	0.48	
Butyric	0.60	0.60	
Isovaleric	0.76	0.74	
Crotonic	0.84	0.83	
Valeric	1.00	1.00	
Isocaproic	1.42	1.42	
Caproic	1.68	1.68	
Heptylic	2.94	2.90	
Caprylic	4.82	4.82	
Pelargonic	8.12	8.16	
Capric	1.00	1.00	175
Lauric	2.21	2.25	
Myristic	5.50	5.51	
Palmitic	12.0	12.1	

Table 2. Relative retention times of ethyl esters of aliphatic carboxylic acids from a bread pre-ferment and a synthetic acid mixture.

Parent acid	Relative retention time: Synthetic mixture	Relative retention time: Pre-ferment	Column temperature (°C)
Formic	0.11	0.11	50
Acetic	0.17	0.18	
Propionic	0.31	0.31	
Isobutyric	0.40	0.40	
Butyric	0.59	0.60	
Isovaleric	1.00	1.00	
Crotonic	1.20	1.21	
Valeric	1.39	1.41	
Isocaproic	0.83	0.86	75
Caproic	1.00	1.00	
Heptylic	1.50	1.49	
Caprylic	2.39	2.39	
Pelargonic	3.94	3.93	
Capric	6.61	6.60	
Lauric	1.00	1.00	175
Myristic	2.07	2.06	
Palmitic	4.60	4.58	

ACKNOWLEDGMENT

The authors thank Dr. Walter S. Hale for preparing the pre-ferment samples used in this study.

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Fatty Acid Composition of Meat Tissue Lipids

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SUMMARY

The lipids extracted from beef and pork muscle were fractionated into triglycerides, cephalins, and a mixture of lecithins and sphingomyelins. The fatty acid composition of these fractions was determined, and the possible effect of phospolipids on meat flavor was evaluated.

Cold-water extracts of lean beef and lean pork contain desirable meat-flavor precursors. These extracts do not, however, contain any appreciable proportion of the lipids present in the lean meat. Lipids, particularly the phospholipids, are among the more unstable constituents of lean meat; and Younathan and Watts (1960) have recently suggested that the phospholipids play a major role in accelerating flavor deterioration in cooked meats. This paper is concerned with the effect on flavor of the lipids present in lean meat prior to preparation for consumption. Our studies have therefore been made on aged lean beef and lean pork. The meat tissue lipids have been separated into neutral lipids and phospholipids, the fatty acids present in these fractions determined, and the possible contribution of these fractions to either desirable or undesirable flavor evaluated.

EXPERIMENTAL

Extraction of lipids from muscle. As in previous flavor studies (Hornstein and Crowe, 1960; Hornstein *et al.*, 1960b), fresh meat was aged 10 days at 36–38°F. Several of the muscles were then dissected and stored at 0°F. As needed, samples of meat were thawed, fat was removed, and the trimmed muscle was cut into small sections. The extraction procedure was essentially that of Folch *et al.* (1957). One hundred grams of the diced meat were blended for 5 min with 900 ml of cold 2:1 chloroform-methanol (all solvents are reagent grade and all solvent ratios are v/v) in an Oster blender (mention of trade names is for identification and implies no endorsement). The slurry was immediately filtered, then mixed with 0.2 its volume of water in a 2-L separatory funnel, and allowed to stand overnight at 3–5°C. The separation into two phases was clean-cut. The lower phase was drained and, without further washing, dried over anhydrous sodium sulfate, concentrated to a small volume on a rotary evaporator at room temperature under partial vacuum, quantitatively transferred with several ml of chloroform to a tared 125-ml Erlenmeyer flask, and dried on the rotary evaporator. Residual solvent was removed under high vacuum. The weighed residue was redissolved in 20 ml of 20:1 chloroform-methanol. Small amounts of undissolved material were removed by centrifugation.

Separation of neutral fat from phospholipids by column chromatography. Fifty grams of silicic acid (Mallinckrodt AR 100-mesh) heated overnight at 130°C were slurried with chloroform and poured into a 2.5 × 90-cm column fitted with a sintered-glass disc. Air bubbles were removed by stirring the mixture with a long glass rod. The silicic acid was allowed to settle and the chloroform drained under slight nitrogen pressure. When the column was compact and with at least 15 cm of liquid above the interface, anhydrous sodium sulfate was added to form a 2.5-cm-thick layer on top of the silicic acid. The column, after washing with 300 ml of chloroform, was ready to use. The entire lipid sample, containing as much as 1 g of phospholipid and 7 g of neutral fat, was added. The column was developed with successive 300-ml portions of chloroform-methanol 20:1, chloroform-methanol 1:1, and methanol. Flow rates of approx 3–5 ml/min were obtained by applying 5 lb of nitrogen pressure to the top of the column. Fractions collected were dried as described for the

total lipid fraction, and the residues were weighed.

Analytical methods. Each fraction was made up to 50 ml with the solvent mixture used for its elution, and aliquots were taken for the determination of phosphorus, total and amino nitrogen, reducing sugars, and fatty acid composition. The infrared spectrum was also recorded. *Phosphorus* was determined after wet oxidation of an aliquot containing 1–10 μg of phosphorus. The phosphate formed was measured by reduction of the phosphomolybdate complex according to the procedure of Chen *et al.* (1956). *Total nitrogen* was determined by the micro-Kjeldahl procedure with mercuric oxide as the catalyst. *Amino nitrogen* was determined by the ninhydrin procedure of Moore and Stein (1948) as modified by Lea and Rhodes (1954). *Reducing sugars* were determined by the anthrone method used by Radin *et al.* (1955) in determination of cerebrosides. *Infrared spectra* were obtained by deposition of a film of the material by solvent evaporation on sodium chloride plates and scanning the region of 2.5–15 μ. The instrument used was a Perkin-Elmer 137 Infracord spectrophotometer. *Fatty acid determinations* were made on aliquots taken to dryness and containing 100–200 mg of material. Twenty-five ml of 0.5*N* alcoholic KOH were added to each residue and the mixture gently refluxed for 6 hr. The contents of the flask were transferred to a 125-ml separatory funnel, 50 ml of water were added, and the solution was made acid to phenolphthalein by dropwise addition of concentrated HCl and then 1 ml excess added. The solution was extracted with three successive 25-ml portions of petroleum ether, and the combined ether extracts were washed three times with 25-ml portions of cold distilled water. At this point, approx 20 mg of *n*-heptadecanoic acid (recrystallized twice from methanol), accurately weighed, were added to the solution to act as an internal standard in gas chromatographic analysis of the fatty acid esters (Hornstein *et al.*, 1960a). The fatty acid methyl esters were prepared as previously described, and dissolved in 1 ml of petroleum ether. An appropriate aliquot, containing approx 500 μg of total esters, was used for gas chromatography. The gas chromatograph was a Beckman GC-2 equipped with a 1-mv recorder, and the detector was a 4-filament thermal conductivity unit. The column was an 8-ft. × ¼-in OD coiled Cu tube containing polyvinylacetate on acid and alkali washed Chromosorb R, 15:85 w/w. The operating temperature was 221° and the He flow rate 100 ml/min. Quantitative results were based on recoveries of the C_{17} acid.

RESULTS AND DISCUSSION

Solvent systems. Initial attempts to separate the phospholipids by precipitation with acetone and magnesium chloride left phosphorus in solution. To avoid phospholipid losses, acetone precipitation was discarded and chromatography on silicic acid used (Borgström, 1952). The elution solvents were chloroform-methanol mixtures (Hanahan *et al.*, 1957); increasing the percent methanol in chloroform by small increments resulted in smeared bands. The sharpest separations were obtained starting with 20:1 chloroform-methanol (Solvent I), followed by 1:1 chloroform-methanol (Solvent II), and finally eluting with anhydrous methanol (Solvent III). Each of the eluted fractions contained a mixture of lipids in which one class predominated. Solvent I eluted the nonphosphorous-containing lipids. This eluate was collected as two fractions. Fraction Ia contained 85–90% of the neutral lipid fraction; collection was stopped prior to the emergence of a heavily pigmented band. Fraction Ib contained these pigments as well as neutral lipids. Silicic acid and Solvent I had similar refractive indices, and the column was translucent. The progress of Solvent II through the

Table 1. Chemical analysis of fractions obtained by silicic acid chromatography of muscle lipids.

Fraction	Phosphorus (%)	Total nitrogen (%)	Amino nitrogen (%)	Reducing sugars	Infrared absorption at 10.3 μ	Major components
Ia	0.0	.....	0.0	.....	.....	Triglycerides
Ib	0.1	.....	0.0	weak, positive	.....	Triglycerides
II	3.6	1.7	1.7	.....	absent	Cephalins
III	4.0	1.9	0.1	.....	strong	Lecithins and sphingomyelins

column caused the column to become progressively opaque. Just before Solvent II broke through, Fraction II was collected; the last 150 ml of Solvent II eluted only trace amounts of material. Methanol eluted the final fraction. Further elution with methanol, plus 5% water, yielded only traces of material. This same elution pattern was obtained with lipid extracts from both lean beef and lean pork.

Classification of fractions. In order to classify these fractions, extracts from several muscles were analyzed. The data fell into a similar pattern for all muscles; a typical analysis is shown in Table 1. Fractions Ia and Ib, as mentioned, were predominantly triglycerides, and for the purpose of this study were not further characterized. The weak positive tests for phosphorous and reducing sugars suggested the presence of small amounts of phospholipids and cerebrosides in Fraction Ib. Infrared spectra of Fractions II and III gave typical phospholipid spectra (Nelson and Freeman, 1959; Smith and Freeman, 1959). Without pure reference standards, slight variations in spectra cannot be interpreted properly. The major difference between II and III resided in the 10.3-μ region (Fig. 1). The

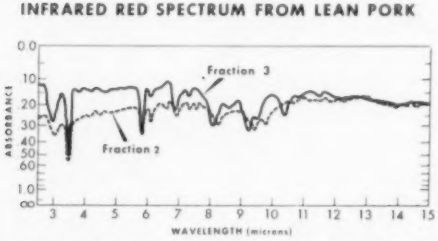

Fig. 1. Infrared spectrum of Fractions 2 and 3 obtained from pork.

absence of absorption at 10.3μ in Fraction II was indicative of cephalins, and the strong absorption in Fraction III at this wave length was characteristic of lecithins and sphingomyelins (Nelson and Freeman, 1959; Smith and Freeman, 1959). Fraction II had the expected 1:1:1 molar ratio of amino nitrogen/total nitrogen/phosphorus. However, cephalins accounted for only about 90% of this fraction since these analyses were 10% lower than expected. The very weak test for amino nitrogen in Fraction III excluded cephalins except in trace quantities. Total nitrogen was 1.9% (comparable figures are lecithins 1.7% and sphingomyelins 3.0%). The observed value indicated about 15% sphingomyelins in Fraction III.

The phospholipids in Fractions II and III were associated with protein. Folch and Lees (1951) described a class of lipoproteins that had the solubility characteristics of lipids; when dried, even under the mildest conditions, these lipoproteins yielded an insoluble protein fraction. The cephalin fraction we obtained contained about 20% of this "proteolipid" material. For example, a typical Fraction II, weight 331.8 mg, was dried and then redissolved in Solvent II, and 50.3 mg of residue remained. Successive dryings yielded 8.2 mg and 4.0 mg of residue. Analysis of this residue showed no phosphorus and 6.1% nitrogen. Fraction III typically contained about 2% of this proteinaceous material. This protein denaturation is minimized in the presence of fat since the total extract, when taken to dryness, did not exhibit this phenomenon to as great an extent. Our analytical data are reported on the basis of protein-free phospholipids.

Fatty acid determinations. Table 2 tabulates the lipid fractions extracted from typical samples of beef and pork. In general, beef muscle contained 2.0–4.0% of nonphosphorous-containing lipids, and pork muscle contained 5.0–7.0%. The phospholipid content was 0.8–1.0% in beef and 0.7–0.9% in pork. The approximate composition of the phospholipid fraction for both beef and pork was cephalin 40–45%, lecithin 40–45%, sphingomyelin 10–15%, and protein 5–10%. Table 3 shows the fatty acid composition of the fractions isolated from these samples. The absolute recoveries of fatty acids in Fractions Ia and Ib, based on 90% fatty acid content, were about 95%. Recoveries based on 75% fatty acid content for protein-free Fractions II and III were respectively approx 50 and 80%. In part, these low recoveries can be attributed to the presence of plasmalogens as evidenced

Table 2. Weight in grams of lipid fractions isolated from 100 g of muscle by chloroform-methanol extraction, followed by silicic acid chromatography.

	Beef	Pork
Total lipids	4.5699	6.6098
Non-phosphorus lipids	3.5539	5.8615
Fraction Ia (triglycerides)	2.8962	5.4473
Fraction Ib (triglycerides)	0.6577	0.4142
Phospholipids	1.0040	0.7462
Fraction II (cephalins)	0.4330	0.3155
Fraction III (lecithins and sphingomyelins)	0.4990	0.3737
Denatured proteins	0.0720	0.0570
Total recovery	4.5489 (99.5%)	6.6077 (99.9%)

by the formation of 2, 4-dinitrophenylhydrazones of long-chain carbonyl compounds after mild acid hydrolysis of these fractions, and to the formation of oxidized phospholipids as shown by the characteristic ultraviolet absorption at approx 235 mμ and 275 mμ (Lea, 1957).

The identities of the saturated acids and oleic, linoleic, linolenic, and arachidonic acids were established by direct comparison of retention volumes of the methyl esters with those of authentic specimens. The other assignments were based on plots of the log of retention volumes versus the number of carbon atoms. Saturated fatty acid methyl esters fall along a straight line—those containing one double bond along a parallel line, those with two double bonds along a second parallel line, etc. (Hornstein *et al.*, 1960a). As the number of double bonds in the molecule increases, the distance between these parallel lines tends to decrease; and it is possible, for example, that the "envelope" assigned to arachidonic acid may contain higher unsaturated C_{20} acids. The assignment made for tetradecenoic, tetradecadienoic, palmitoleic, eicosatrienoic, and docosadienoic should be considered rendered highly probable rather than absolute, since they were not based on direct comparison with authentic samples. Table 4 shows the fatty acid composition of the combined triglyceride fractions and of the combined phospholipid fractions. Unsaturated acids containing two or more double bonds make up 10% of the triglyceride fraction and about 50% of the phospholipid fraction; however, the actual milligrams of these acids contributed by the two fractions are similar since the ratio of triglycerides to phospholipids is about 4 : 1 in beef and about 8 : 1 in

Table 3. Fatty acid composition of lipid fractions (% of total fatty acids) obtained from lean beef and pork. Fractions Ia and Ib triglycerides, Fraction II cephalins, Fraction III lecithins and sphingomyelins.

	Fraction Ia		Fraction Ib		Fraction II		Fraction III	
Acid	Beef	Pork	Beef	Pork	Beef	Pork	Beef	Pork
Capric	.....	.....	1.1	2.7	.....	.....	.....	.....
Lauric	.....	0.2	0.6	0.4	.....	.....	.....	.....
Myristic	2.1	1.1	2.8	2.0	1.4	3.6	3.6	1.0
Tetradecenoic	1.0	.....	1.5	.....	0.6	0.1	1.1	0.3
Tetradecadienoic	0.6	.....	0.6	.....	0.6	0.8	1.3	0.5
Palmitic	28.3	24.0	19.8	22.5	2.8	4.1	21.0	30.5
Palmitoleic	4.6	7.2	5.9	9.8	1.8	2.4	2.4	2.4
Stearic	17.0	12.1	16.2	7.0	27.4	20.6	6.6	4.6
Oleic	42.5	45.8	30.6	39.4	13.8	14.3	26.9	17.4
Linoleic	3.0	7.9	17.2	15.3	16.2	15.2	23.6	36.3
Linolenic	0.9	1.7	2.5	1.7	2.1	0.4	1.7	1.4
Eicosatrienoic	.....	.....	.....	.....	.....	4.0	3.2	.....
Arachidonic	.....	.....	1.2	1.2	33.3	32.4	8.6	5.6
Docosadienoic	.....	.....	.....	.....	.....	2.1	.....	.....
Weight of fraction (g)	2.8962	5.4473	0.6577	0.4142	0.4330	0.3155	0.4900	0.3737

Table 4. Fatty acid composition of combined triglyceride fractions and of combined phospholipid fractions from Table 3 (% of total fatty acids).

Acid	Triglycerides		Phospholipids	
	Beef	Pork	Beef	Pork
Capric	0.1	0.1	....	....
Lauric	0.1	0.2	....	....
Myristic	2.2	1.2	2.6	2.0
Tetradecenoic	1.0	....	0.9	0.2
Tetradecadienoic	0.6	....	1.3	0.6
Palmitic	27.5	23.9	13.2	20.0
Palmitoleic	4.7	7.4	2.2	2.3
Stearic	16.9	11.6	15.6	11.0
Oleic	41.3	45.2	21.2	16.2
Linoleic	4.4	8.7	20.2	27.9
Linolenic	1.1	1.6	1.8	1.0
Eicosatrienoic	....	....	1.8	1.6
Arachidonic	0.1	0.1	19.2	16.3
Docosadienoic	....	....	....	0.9
Total saturated acids	46.7	37.0	31.4	33.0
Total monounsaturated acids	47.1	52.6	24.3	18.8
Total dienoic acids	5.0	8.7	21.5	29.3
Total trienoic acids	1.1	1.6	3.7	2.6
Total tetraenoic acids	0.1	0.1	19.1	16.3

pork. The major quantitative difference is the large amount of arachidonic acid (possible maximum 150 mg per 100 g of muscle in beef and 100 mg/100 g of muscle in pork) contributed by the phospholipid fraction that has no counterpart in the triglyceride fraction (Table 3).

Flavor characteristics. We attempted to evaluate the aromas developed by heating freshly isolated lipid fractions in air. The odors of the beef and pork triglyceride fractions were respectively reminiscent of "fried-fat" and "bacon." The cephalin fractions from both beef and pork produced strong "fishy" odors, probably attributable to the high arachidonic acid content of these fractions. The heated lecithin-sphingomyelin fractions from both beef and pork were also alike in odor, the "fishy" smell less pronounced than in the cephalin fraction and superimposed on an aroma suggestive of liver. Total lipid and phospholipid extracts of pork and beef were exposed to air, and their odors noted at 24-hr intervals. Rancid odors developed more quickly in these samples than in neutral fat. At the end of one week, the samples were all highly rancid. The odor of the pork total lipid extract was the least objectionable; the large amount of triglyceride present either dissolved the potent odor compounds produced, thus lowering their vapor pressure, or mechanically inhibited the oxidation of the phospholipids by limiting their surface exposed to air. It was concluded that phospholipids did not contribute to desirable meat flavor and that the possibility existed that in excessively lean meat they could contribute to poor flavor. The triglyceride contribution was similar to that previously described (Hornstein and Crowe, 1960). Changes in color were also noted in these fractions. The phospholipids darkened rapidly, the total lipid fraction changed color more slowly, and the neutral lipids showed the least color change.

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Temperature and the Retrogradation of Starch

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(Manuscript received June 27, 1961)

SUMMARY

Cornstarch sols (2%) were prepared at a series of gelatinization temperatures (80–127°C) and stored at 1, 37, and 70°C. At 1°C storage, retrogradation was slower as the temperature of gelatinization was greater. At 37°C storage, no retrogradation occurred in samples gelatinized at lower temperatures, and solubilization took place in samples gelatinized at higher temperatures. At 70°C storage, solubilization occurred in all samples, but more rapidly as the temperature of gelatinization was greater.

It has generally been found that retrogradation (i.e., decreasing solubility and increasing crystallization) of a starch paste is more or less inhibited at holding temperatures in the vicinity of 60°C and that it tends to be accelerated with decreasing temperature (Katz and Rientsma, 1930; Katz and Weidinger, 1934; Maquenne, 1903, 1904; Whistler, 1954). According to Lampitt *et al.* (1941), however, retrogradation is the same at 37°C as at 62°C in 2% pastes of fractions of thoroughly milled wheat starch; and at lower paste concentrations, retrogradation at 21°C is equal to that at 37 and 62°C. Results of Lampitt *et al.* (1948) were essentially similar with potato starch fractions. Likewise, Hull and Schoch (1959) showed little difference in solubility between starch films dried at 80°C and those dried at 30°C. Sterling (1960) recently reported that 13% starch gels held at 70°C become less dispersible than those held at room temperature. Thus, there are clearly discrepancies in reports on the role of storage temperature in retrogradation.

A few studies have been directed to the relationship of retrogradation with temperature of gelatinization. Maquenne (1904) showed that retrogradation in pastes of potato starch was less in those gelatinized at 120°C than in those gelatinized at 110°C, and less in those gelatinized at 110°C than in those gelatinized at 100°C. Comparable findings were reported by van Itallie (1930), who stated that, with gelatinization temperatures of 120, 100, and 65°C, retrogradation was greater the lower the temperature. No other systematic studies have been performed.

Because of this fragmentary or controversial state of knowledge, it was believed fruitful to explore further the relation between temperature and the retrogradation of starch. Both temperature of gelatinization and temperature of storage are variables in this study. The authors have elected to use 2% cornstarch sols as raw material and to measure retrogradation by the sensitive techniques of cold-water solubility and enzymatic digestibility. To some extent, a new X-ray diffraction method has also been essayed.

MATERIALS AND METHODS

Slurries were made of 2% native cornstarch in water, neutralized to pH 7 with ammonium hydroxide. The starch was gelatinized by heating the slurry for 10 min in a round-bottom flask equipped with a stirrer, condenser, and thermometer. (This arrangement made it possible to prevent the formation of a surface film.) The heating temperatures were 80, 90, and 100°C. When the temperatures used were higher than 100°C (110, 120, and 127°C), the slurries were heated to 100°C in the above-described flask and immediately transferred to an autoclave for further heating. The additional heating periods were 10 min for 110 and 120°C, and 30 min for 127°C.

* This study was supported in part by the U. S. Public Health Service, under Grant RG-5726. For this help the authors are truly thankful.

The starch solutions were then stored in tightly capped vials with 5 drops of toluene added for preservation. Storage temperatures were 1, 37, and 70°C for each gelatinization temperature.

The determination of water-soluble starch, both by iodine adsorption and by hydrolysis of the supernatant with HCl, has been described (Kalb and Sterling, 1961). Similarly, enzymatic hydrolysis of the whole sol was carried out by the technique given in that same report.

X-ray measurements, following the method of Hermans and Weidinger (1949), were made to ascertain the relative crystallinity of the starch material. The rationale of this approach is the presumption (Meyer, 1952; Schoch, 1952; Sterling, 1957) that retrogradation is essentially a process of crystallization of the starch molecules. However, the amorphous background of the photometric traces was quite large relative to the size of the small crystalline peaks, and the areas of the latter could be determined only with difficulty.

A new X-ray method was developed that proved useful in ascertaining the rate of crystallization. It has been demonstrated that a freshly prepared starch paste shows a so-called V-pattern when precipitated with alcohol (Bear, 1952; Katz, 1930; Wiegel, 1941). During retrogradation, the alcohol-precipitated paste gradually displays an increasing intensity of the B-pattern (Katz, 1930) and a decreasing intensity of the V-pattern.

Consequently, a 50-ml portion of the starch sol was precipitated with 16 volumes of 95% ethanol, filtered, washed with fresh ethanol, and dried at room temperature under vacuum. The dry powder was packed in a sample holder with a drop of 2% commercial resin (Harleco) in toluene and exposed to an X-ray beam (Cu-Kα, monochromatized with a crystal of pentaerythritol) collimated to 0.8 mm diameter. A film in a flat cassette, 4.0 cm from the sample, was used in the production of a photometric trace of the scattered intensity. In this trace, both the major V-peak ($d = 6.9$ Å) and the strong B-peak ($d = 5.4$ Å) are individualized at an early stage in retrogradation, being most distinct at about 5 days. (Before 5 days, the B-pattern is usually not obvious, and after 5 days V-patterns in more rapidly retrograding samples tend to be indistinct). The heights of the two peaks of interest were evaluated, at their maxima, above the interpolated amorphous background. The index of retrogradation is given as the ratio of the height of the B-peak to that of the V-peak (B/V).

RESULTS

The relative effect of gelatinization temperatures is readily shown in Fig. 1. Here is represented the ratio of the B- to V-peak heights after 5 days' storage at 1°C. It is apparent that the lower the temperature of gelatinization, the greater the relative amount of subsequent retrogradation.

A feature of interest is shown in the course of retrogradation during the 28-day period of storage. Fig. 2 illustrates the change in content of soluble starch as measured by hydrolysis. It may be seen that the amount of soluble starch is initially a function of the temperature of gelatinization. During storage, retrogradation occurs in all specimens. The trends of decreasing soluble starch content seem to be approaching a common level, with that level attained earlier with the lower gelatinization temperatures. In fact, starch gelatinized at 127°C apparently is highly soluble for about 9 days, after which it retrogrades rapidly. The same trends are shown in the analysis of soluble starch via iodine adsorption (Fig. 3) and in the enzymatic digestibility of the whole sol (Table 1).

At storage temperatures of 37 and 70°C the relative retrogradative tendencies of samples gelatinized at different temperatures are maintained. Samples gelatinized at 80°C have the greatest

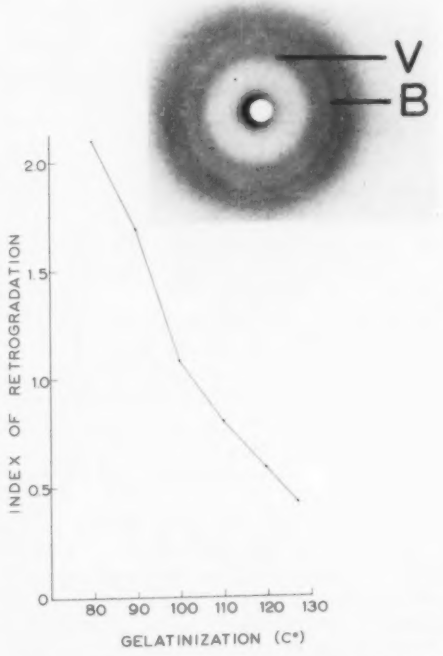

Fig. 1. Retrogradation, as measured by B/V ratio, at 5 days in starch sols held at 1°C after gelatinization at different temperatures. Inset shows typical scattering pattern, with B and V peaks indicated.

Table 1. Content of maltose (γ/ml) produced by enzymatic hydrolysis of 2% corn starch sols stored at 1°C.

Gelatinization temperature (C°)	Time of storage (days)				
	0	2	5	9	28
80	690	570	520	480	460
90	670	590	540	500	450
100	700	600	580	530	490
110	670	600	620	540	480
120	690	600	630	580	500
127	740	680	680	640	560

amount of retrogradation, and samples gelatinized at 130°C have the least, with samples gelatinized at intermediate temperatures exhibiting intermediate retrogradation. Actually, as is shown below, storage at higher temperatures results in a reversal of retrogradation, i.e., solubilization.

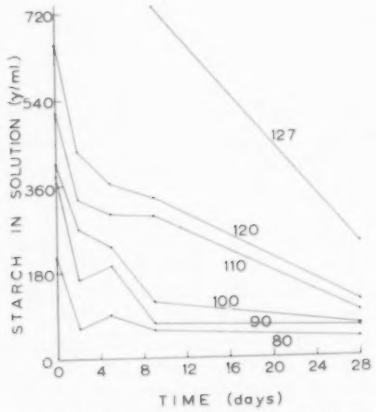

Fig. 2. Soluble starch content in sols held at 1°C, after gelatinization at different temperatures as indicated. Soluble starch determined via HCl hydrolysis of the supernatant material. (Limit of sensitivity of method is at upper edge of graph.)

The effect of 37°C storage is indicated in Fig. 4. Here it may be seen that, in general, no retrogradation occurs. With lower temperatures of gelatinization, the starch sol remains more or less unchanged, or experiences a slight solubilization. With increased gelatinization temperature the solubilization effect is more definite.

During storage at 70°C, solubilization is marked in all the samples, and both the solubility of the starch and its enzymatic digestibility are increased. Again, the higher the temperature of gelatinization the greater is the extent of solubilization (Fig. 5).

DISCUSSION

As a preface to the discussion of the general results, it is well to emphasize that the role of gelatinization temperature may be intermingled, to some extent, with the role of heating time. Because of the experimental difficulties it was not possible to eliminate the differences in time involved in bringing the starch pastes to the required temperatures of gelatinization. However, Katz (1928) has reported that doubling the period of holding a starch paste at a particular gelatinization temperature seems to be without additional effect on the swelling properties or the solution properties of the starch.

When a 2% cornstarch sol is stored at 1°C, it is quite evident that the retrograding tendency is stronger the lower the temperature of gelatinization. Perhaps the simplest explanation is, in agreement with the principle of Occam's razor, the correct one: the lower the temperature of gelatinization the less the dispersal of the starch molecules. In fact, at 80°C, which is just barely over the specific gel point for native starch, it might be supposed that rather large extents of the molecules are still held together in micellar regions. The higher the gelatiniza-

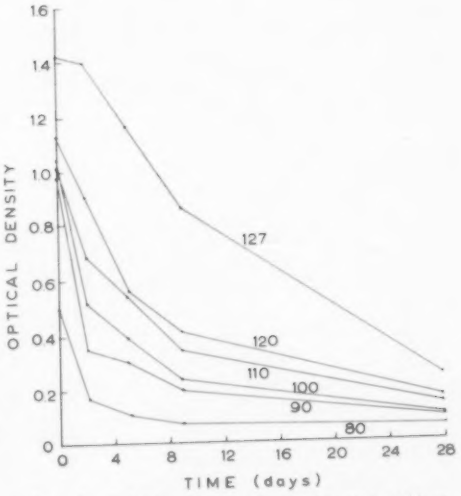

Fig. 3. Soluble starch content in sols held at 1°C, after gelatinization at different temperatures as indicated. Soluble starch determined via iodine adsorption of the supernatant material.

tion temperature the more dispersed are the starch molecules, which eventually become free in solution (*cf.* Katz, 1928). Thus, it is justifiable to imagine that the starch molecules can associate in a much shorter time when they are closer together than when they are farther apart. Moreover, when micellar regions are already present, aggregation proceeds faster: Holló *et al.* (1959) have shown that the presence of such crystalline domains facilitates renewed molecular association when a starch sol is cooled, in the manner of crystalline nuclei in supersaturated solutions.

In this way, starch gelatinized at lower temperatures will aggregate more rapidly than will starch gelatinized at higher temperatures. The common level toward which all solutions would be tending is perhaps the original state of association within the starch grain—hence the validity of the term "retrogradation." Note that a sort of "lag" period exists for the sample gelatinized at 127°C. This may be the period necessary for nucleation centers to reach a stable size and thereby initiate rapid crystallization.

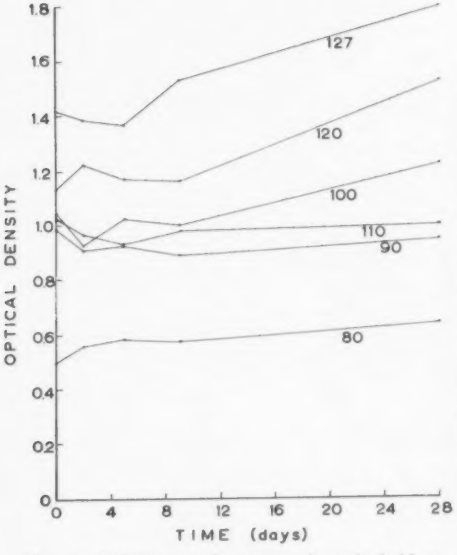

Fig. 4. Soluble starch content in sols held at 37°C, after gelatinization at different temperatures as indicated. Soluble starch determined via iodine adsorption of the supernatant material.

When the sols are held at higher storage temperatures, the same order of the effect of gelatinization temperature obtains. In the storage temperatures used here, the ranking of gelatinization temperatures from higher to lower values is related to decreasing dispersion or decreasing solubilization during storage. It is evident that, in agreement with the data of earlier workers, little retrogradation occurs during storage at 37°C. In fact, a slight solubilization effect has been documented.

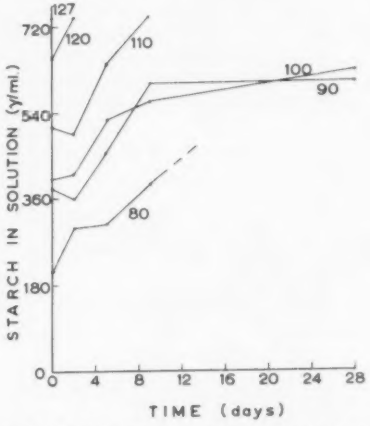

Fig. 5. Soluble starch content in sols held at 70°C, after gelatinization at different temperatures as indicated. Soluble starch determined via HCl hydrolysis of the supernatant material. (Limit of sensitivity of method is at upper edge of graph.)

At 70°C all samples become more and more solubilized. Maquenne (1904b) reported a similar effect with partially retrograded starch pastes. Katz (1928), however, stated merely that at 60°C there is no retrogradative tendency. Obviously, effective hydrogen-bond rupture is occurring in the starch sol at 70°C (*cf.* Katz and Weidinger, 1934). Roux (1905) and others believed that this could take place only at significantly higher temperatures, at which, however, the possibility of hydrolytic scission of the glucosidic linkages is greatly enhanced.

These results differ markedly from those of Hull and Schoch (1959) and Sterling (1960). The discrepancy is possibly due to the fact that the latter observers worked with

much more concentrated systems. When concentrated starch systems are heated, the X-ray diffraction pattern of the resulting crystallites is near-*A* (*C*) or *A* (Bear and French, 1941; Katz and Derksen, 1933; Sair and Fetzer, 1944; Sterling, 1960). This is in agreement with the evidence that *A*-starch has a lower degree of hydration than does *B*-starch (Badenhuizen, 1959; Hellman *et al.*, 1954). Also, according to Badenhuizen (1959), starch that is associated in *A*-crystallites is more rigid and swells less than the same starch in *B*-crystallites. This conception will explain the discrepancy noted above as well as the results of Whittenberger and Nutting (1948) with potato starch heated in the presence of limited moisture.

An additional possibility, of probably lesser consequence, is that the soluble amylopectin-amylose ratio increases continually with increasing gelatinization temperature (or increases at a higher storage temperature as the time of storage increases). This result could presumably bring about a concomitant retardation of molecular association in solution—if the effect of dissolved longer amylose chains did not work in the opposite direction. However, it is to be recalled that amylopectin molecules also do participate in retrogradation (Schoch, 1952), and hence may not exercise a marked hindrance to that process (at least in the proportions present in corn starch).

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Effect of Temperature and Holding Period on Some Physical and Chemical Characteristics of Lemon Fruits [a]

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(Manuscript received August 18, 1961)

SUMMARY

Changes were followed in fresh weight, juice content, soluble solids, total acidity, and ascorbic acid of lemon fruits during 4, 8, and 12 weeks at 4, 13, and 24°C and after 3 and 14 days at 24°C following 4, 8, and 12 weeks at 4 and 13°C. Juice content, soluble solids, and total acidity increased during storage. The increase was greatest for fruit held at 24°C and least for fruit held at 4°C. Upon transfer to 24°C from 4 and 13°C, all three constituents increased. Changes in ascorbic acid in the lemon fruit do not appear related to the mechanism of chilling injury, but the losses observed in chilled fruit when transferred to 24°C were secondary, probably associated with symptom development.

INTRODUCTION

Most of the lemon crop is picked during periods when the commercial demand is light. Therefore, lemon fruits are stored for various periods, depending on their condition and market demands. Lemon fruits are normally harvested by size rather than color or maturity. In addition, this storage period is desirable because lemon fruits do not produce the maximum amount of juice or the highest quality of juice when picked, but require conditioning to develop color, juice content, and flavor. The literature on storage conditions and changes in the lemon fruit during storage has been reviewed (Bartholomew and Sinclair, 1951; Miller, 1946, 1958; Rose *et al.*, 1951; Wright *et al.*, 1954).

In general, the optimum storage conditions and some of the physical and chemical changes occurring in lemon fruit during storage are known (Bartholomew and Sinclair, 1951; Miller, 1946, 1958; Rose *et al.*, 1951; Wright *et al.*, 1954), whereas compositional changes in relation to storage temperature have not been fully evaluated. Of special interest is the influence of lower storage temperatures, particularly in the chilling range. Ascorbic acid has been discussed in relation to chilling injury for other crops. With bananas (Harris and Poland, 1939), pineapple (Miller, 1951; Miller and Heilman, 1952) and sweet potato (Lieberman *et al.*, 1958, 1959) a loss of ascorbic acid has been associated with chilling injury. However, changes in ascorbic acid content of tomato fruits did not appear to be related to chilling injury (Craft and Heinze, 1954).

Reported here are observations on some physical and chemical changes occurring in lemon fruits during 4, 8, and 12 weeks of storage at 4°, 13°, and 24°C and during 3 and 14 days at 24°C following 4, 8, and 12 weeks at 4° and 13°C.

MATERIALS AND METHODS

Freshly harvested and processed light-green Eureka lemon fruits (*Citrus limonia* Osbeck) were obtained from commercial packing houses in the coastal and interior lemon-producing areas of California. The bulk supply from each area was divided into random samples of 20 fruits each, weighed, and placed in paper bags, and the bags were placed in lemon storage boxes and put in storage, except for samples from each area that were analyzed immediately. All storage treatments from each area were replicated eight times. The storage treatments consisted of 4, 8, and 12 weeks

[a] Paper No. 1317, University of California Citrus Experiment Station, Riverside, California.

at 4, 13, and 24°C and 3 and 14 days at 24°C following 4, 8, and 12 weeks at 4 and 13°C. These storage temperatures cover the range from low temperature (4°C), reported to be injurious, to optimum storage temperature (13°C), to marketing temperature (24°C).

Two sets of samples from each treatment and replication were used; one was juiced and the other was peeled and the peel and pulp analyzed separately. Characteristics investigated were: a) appearance, both internal and external; b) incidence of decay; c) fresh-weight changes of the whole fruit, rind, and pulp; d) amount of juice reamed from the fruit; e) soluble solids in the juice, corrected for acidity (Stevens and Baier, 1939); f) total titratable acid expressed as anhydrous citric acid; g) pH; h) ascorbic acid content of the juice, rind, and pulp excluding seeds, by a colorimetric method (Loeffler and Ponting, 1942); and i) dry weight of the rind and pulp. The data obtained made possible the expression of the chemical composition either as a concentration, based on the fresh or dry weight at sampling, based on original fresh or dry weight, or on a per-fruit basis. The data for the fruit from the two areas were combined, obviating unwieldy tables and confusion in the figures. The results appeared most relevant when based on concentration and on original fresh weight of the fruit. Presenting the results based on dry weights or on a per-fruit basis would have been redundant. Changes in the fresh and dry weights of the rind and pulp and pH of the juice are not presented, since they do not appear pertinent to the discussion. Statistical analysis, according to accepted techniques, was applied to the data; confidence limits are shown in the figures and tables.

RESULTS AND DISCUSSION

The selection of quality fruit, careful handling, and clean storage conditions did much to suppress decay during storage. Of the nearly 18,000 fruits involved, only 35 decayed (*Penicillium* spp.). Most of these were in samples held 3 and 14 days at 24°C that had suffered chilling injury from 12 weeks at 4°C. Corrections accounting for the decayed fruit were applied to the other data collected.

External appearance remained good with all treatments except for the fruit held 3 and 14 days at 24°C after 12 weeks at 4°C: in addition to decay, it began to show surface pitting, a dull appearance, and discoloration of the buttons, indicating loss of vitality. Also, the internal appearance of these fruits showed signs of physiological breakdown in the form of membranous stain and pathological decay from *Alternaria*. About 25% of the fruits held 14 days at 24° after 12 weeks at 4°C had a translucent, water-soaked appearance when cut.

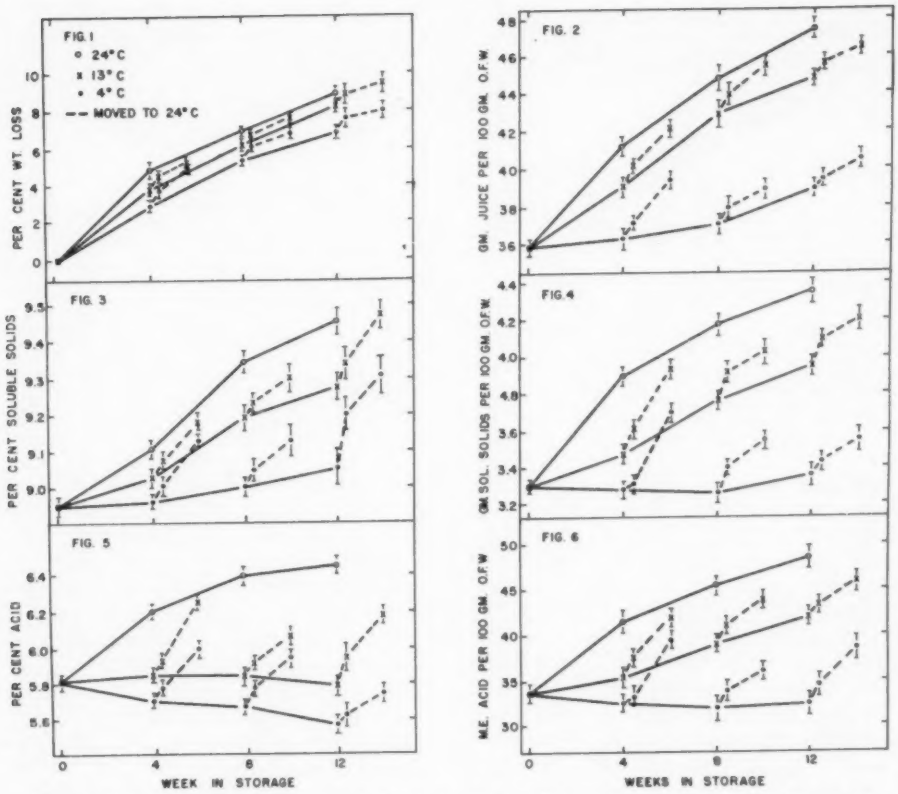
Weight loss is considerable during the first 4 weeks, when the turgidity of the rind was reduced (Fig. 1). In accordance with previous experience the rate of weight loss was a function of the vapor-pressure deficit. Following transfer from 4 and 13°C to 24°C, the fruits lost weight rapidly during the first 3 days, after which the rate of weight loss closely paralleled that of fruit held continuously at 24°C (Fig. 1).

The increase in juice content after the various storage treatments is illustrated in Fig. 2. By expressing the results as grams of juice per 100 grams of original fresh weight of the lemons, the actual changes in the amount of juice reamed from the fruits were determined. Many reports have expressed the results as percentage juice based on the fresh weight of the fruit at the time of sampling. On this basis the percentage juice reamed from the fruits after 0, 4, 8, and 12 weeks at 24°C was respectively 35.9, 43.6, 48.0, and 52.1, which shows the effect of weight loss in exaggerating the increase in juice content.

The source of the increase in reamable juice has been the topic of considerable discussion. The grams change per 100 g of original fresh weight of the fruit in the whole fruit, rind, pulp, and juice (Table 1) indicates that the majority of this increase in juice is derived from changes within the pulp tissue and not a movement of water into the pulp from the rind, as previously reported (Van der Plank *et al.*, 1940); although some increase in pulp weight did occur it was not sufficient to account for the increase in juice.

The increase in soluble solids as percent of the juice (Fig. 3) and grams per 100 g of original fresh weight of the fruit (Fig. 4) reflect the change in concentration (Fig. 3) and the increase in total solids available in the juice (Fig. 4).

Figs. 5 and 6 respectively give the changes in percent total titratable acid as anhydrous citric acid and the millequivalents of titratable acidity in the juice per 100 g of the original



Figs. 1–6. **1)** Weight loss of lemon fruits during 4, 8, and 12 weeks at 4°, 13° and 24°C, and after 3 and 14 days at 24°C following 4, 8, and 12 weeks at 4° and 13°C (broken line). **2)** Juice obtained (grams per 100 g original fresh weight [OFW] of fruit) after storage as described for Fig. 1. **3)** Soluble solids in the juice (percent) after storage as described for Fig. 1. **4)** Soluble solids (grams per 100 g OFW of fruit) after storage as described for Fig. 1. **5)** Titratable acid (percent anhydrous citrus acid in the juice) after storage as described for Fig. 1. **6)** Acid in the juice (millequivalents per 100 g OFW of fruit) after storage as described for Fig. 1.

fresh weight of the fruit. The percent acid in fruits held at 4°C decreased slightly whereas the percent acid in fruits held at 13°C remained essentially constant and fruits held at 24°C showed a progressive increase in percent acid. However, upon removal to 24°C from 4 and 13°C there was a marked increase in the percent acid. The increase in concentration of acid as shown by the percent acid and the increase in juice content results in: a) a considerable increase in total acid available from the fruit held at 24°C, b) moderate increase in fruit held at 13°C, and c) essentially no change in fruit held at 4°C (Fig. 6).

The changes leading to increases in juice content, soluble solids, and acid augment the culinary and processing value of the lemon fruit. Most processors of lemons pay according to the amount of acid per ton of lemons. Likewise the housewife is interested in a maximum of high-quality juice. Lemon fruits entering fresh-fruit channels from storage (13°C) are exposed to temperatures near 24°C during wholesale handling, in the retail store, and in the home to bring about

Table 1. Fresh-weight changes of the whole lemon fruit, rind, pulp, and reamable juice during storage for 4, 8, and 12 weeks at 4, 13, and 24°C per 100 grams original fresh weight (OFW) of the whole lemon fruit.

Storage conditions		Change (g) per 100 g OFW			
Temperature (°C)	Duration (weeks)	Whole fruit	Rind	Pulp	Juice
Initial weight (g/100 g)		100.0	40.7	59.3	35.9
4	4	−2.9	− 2.3	− 0.6	+ 0.5
	8	−5.3	− 4.9	− 0.4	+ 1.1
	12	−6.6	− 6.2	− 0.4	+ 3.0
13	4	−3.9	− 4.3	+ 0.4	+ 3.1
	8	−6.2	− 7.5	+ 1.3	+ 7.1
	12	−8.1	−10.2	+ 2.1	+ 8.8
24	4	−4.9	− 4.9	0.0	+ 5.6
	8	−6.7	− 8.8	+ 2.1	+ 8.9
	12	−8.8	−11.3	+ 2.5	+11.6
LSD	.05	0.7	0.6	0.5	0.6
	.01	0.9	0.8	0.7	0.8

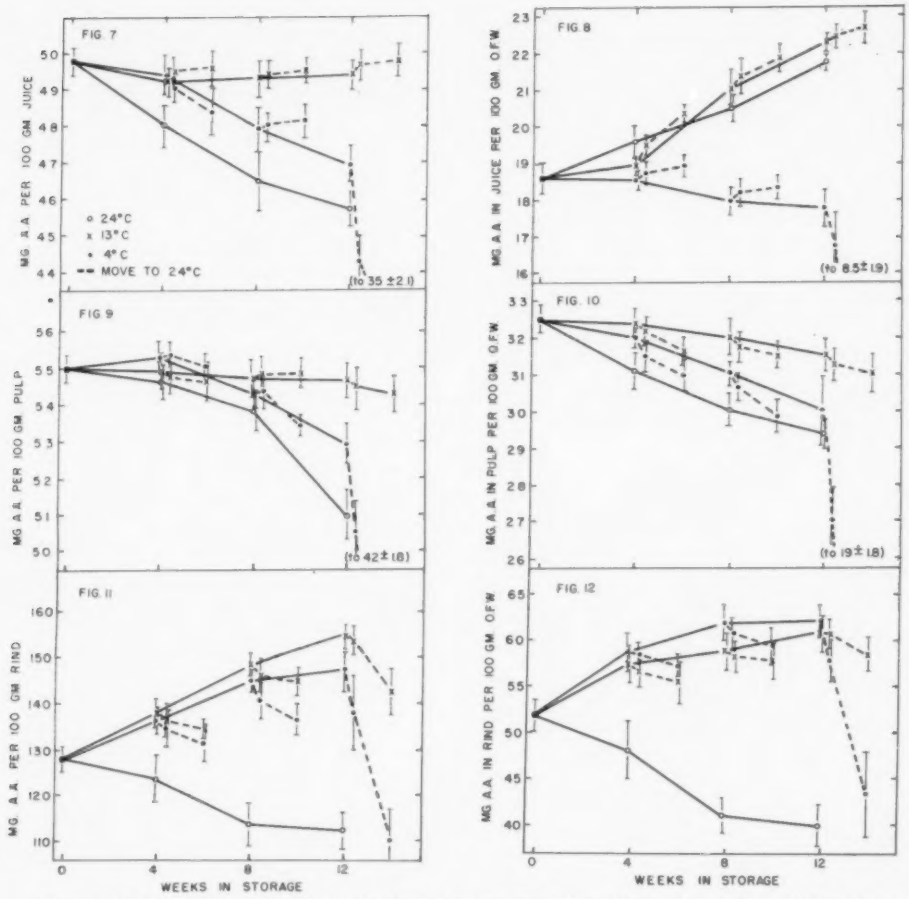
sufficient additional changes to give good quantities and quality of juice. The changes reported here in Figs. 1, 3 and 5 are in general agreement with previous reports and reviews (Bartholomew and Sinclair, 1951; Miller, 1946; Van der Plank *et al.*, 1940). However, previous reports have normally expressed the results in terms of concentration or based upon the fruit weight at the time of sampling. Thus, it is difficult to determine quantitative changes. Expressing the data in terms of quantities per unit of original fresh weight of the fruit (Figs. 2, 4, and 6) presents the actual changes directly.

Changes in the ascorbic acid (vitamin C) content of the juice, pulp, and rind of the lemon fruit are of interest nutritionally as well as physiologically. The milligrams of ascorbic acid per 100 grams of juice (Fig. 7) shows a considerable loss for fruits held at 24°C and, to a lesser degree, for fruit held at 4°C. Fruits held at 13°C maintained about the same concentration of ascorbic acid in the juice. The ascorbic acid concentration in the juice from fruits moved from 4° and 13°C to 24°C showed small but nonsignificant changes, except for those fruits held 12 weeks at 4°C, which showed a drastic loss of ascorbic acid.

Expressing the data as milligrams of ascorbic acid per 100 grams of original fresh weight of the fruit (Fig. 8) presents a completely different picture. The total ascorbic acid found in the juice based on the original fresh weight increased for fruit held at 13 and 24°C and remained essentially constant for fruit held at 4°C. The changes after transfer to 24°C from 4 and 13° were similar to those described for the concentration (Fig. 7).

The concentration of ascorbic acid in the edible pulp of fruit held at 13°C (Fig. 9) was constant during the experimental period. Also, little change occurred after 3 and 14 days at 24°C following storage at 13°C. The pulp of fruit held at 24°C showed small progressive losses in ascorbic acid concentration after 4 and 8 weeks, followed by a considerable decrease in ascorbic acid concentration between the 8th and 12th weeks. The ascorbic acid concentration in the pulp of fruits held at 4°C remained at essentially the same level after 4 weeks, but showed progressive losses from the 4th to 12th weeks. After transfer to 24°C from 4 and 13°C, only minor changes occurred in the ascorbic acid concentration of the pulp, except for the samples held 12 weeks at 4°C, where a considerable loss was observed. The concentration of ascorbic acid in the juice (Fig. 7) and in the pulp (Fig. 9), in general, demonstrates similar trends during storage.

The translucent appearance of the pulp of about 25% of the fruits held 12 weeks at 4°C and then 14 days at 24°C was mentioned previously. This observation indicated that two additional samples should be



Figs. 7–12. **7**) Ascorbic acid (A.A.) in the juice (mg per 100 g juice) after 4, 8, and 12 weeks at 4°, 13°, and 24°C, and after 3 and 14 days at 24°C following 4, 8, and 12 weeks at 4° and 13°C (broken line). **8**) Ascorbic acid in the juice (mg per 100 g original fresh weight [OFW] of fruit) after storage as described for Fig. 7. **9**) Ascorbic acid in the pulp (mg per 100 g pulp) after storage as described for Fig. 7. **10**) Ascorbic acid in the pulp (mg per 100 g OFW of fruit) after storage as described for Fig. 7. **11**) Ascorbic acid in the rind (mg per 100 g of rind) after storage as described for Fig. 7. **12**) Ascorbic acid in the rind (mg per 100 g OFW of fruit) after storage as described for Fig. 7.

taken. Accordingly, after all fruits in each sample had been sampled for ascorbic acid, the normal procedure, a sample was taken from the pulp of the healthy-appearing fruits and from the pulp of the translucent-appearing fruits; the respective average milligrams of ascorbic acid per 100 grams of pulp were 52 and 9 (range 1–15). The healthy-appearing pulp had lost only a small amount of ascorbic acid whereas the translucent, water-soaked-appearing pulp had lost more than 80% of its ascorbic acid during the 14 days at 24°C following the 12 weeks at 4°C. Thus, the normal sampling gave only a modified picture of the true situation. This is a graphic illustration of the selectivity of chilling injury. Three-fourths of similar fruits showed no symptoms of chilling injury whereas the remainder displayed several symptoms (internal discoloration, loss of

semi-permeability resulting in the translucent appearance, susceptibility to decay and breakdown, and, secondarily, loss of ascorbic acid).

The rind of the lemon fruit contains much higher concentrations (Fig. 11) and amounts (Fig. 12) of ascorbic acid than the pulp or juice. The increase in ascorbic acid concentration in the rind during storage at 4 and 13°C (Fig. 11) was not expected. At first it was thought that this was not actual synthesis or a real increase but a reflection of water loss from the rind. However, when based on the original fresh weight of the sample (Fig. 12) it was found that ascorbic acid does, in fact, increase during storage under these conditions. The ascorbic acid content of the rind of samples held 16, 20, and 24 weeks at 13°C showed a gradual decrease to about the initial level by the 24th week. At 24°C the ascorbic acid decreased progressively. Upon transfer from 4 or 13°C the ascorbic acid content decreased in all cases of those chilled (12 weeks at 4°C), again showing a drastic loss in ascorbic acid. For those fruits that were not chilled the decrease in ascorbic acid might be expected since there appears to be two conditions present depending upon temperature, i.e., at 4 and 13°C ascorbic acid appears to accumulate during storage, whereas at 24°C ascorbic acid decreases.

These data definitely indicate that the destruction of ascorbic acid is not the first phase in the development of low-temperature injury, as suggested by Miller and Heilman (1952) from their studies with pineapple. The lemon fruit held 12 weeks at 4°C had suffered chilling injury yet the ascorbic acid content remained near the initial level except in the rind, where an actual increase was observed. Upon removal to 24°C the symptoms of chilling developed (internal discoloration, surface pitting, loss of semi-permeability—resulting in a translucent appearance) and the ascorbic acid content decreased. This resembles results with pineapple (Miller, 1951). Pineapple fruits chilled 48 hours at 5°C and ripened at room temperature contained essentially the same amount of ascorbic acid as fruits ripened without chilling. However, pineapple fruits with physiological breakdown (presumably as the result of chilling injury) had less than 80% as much ascorbic acid as healthy fruits, which is similar to the chilled lemon fruits. The ascorbic acid content of pineapples was about 40% less in those held 7 days at 6°C and 2 days at room temperature than in those held 2 days at room temperature (Miller and Heilman, 1952). Since the ascorbic acid content of the pineapples was not determined at the end of the 7-day chilling period, the decrease observed two days later cannot be considered as the first phase in the development of chilling injury.

Although, from the data presented and the discussion, changes in ascorbic acid did not appear to be associated with low-temperature injury in tomatoes (Craft and Heinze, 1954), a fruit whose chemistry is somewhat similar to that of the lemon, certain analogies appear between the data on tomatoes and the data for lemons presented here. The ascorbic acid content of chilled tomatoes after ripening (19–22 days at 65°F after 4 and 9 days at 32 and 40°F) showed little change and may be similar in degree of injury and response to the 4- and 8-week exposure for lemons to 4°C. However, the tomatoes held 14 days at 32 and 40°F were reported decayed after 18 days at 65°F. Had samples of these fruits been analyzed periodically, beginning immediately after transfer to 65°F, the results may have been similar to those found for lemons upon removal to 24°C after 12 weeks at 4°C, i.e., a rapid decrease in ascorbic acid as the symptoms of chilling develop.

Sweet potatoes (Lieberman *et al.*, 1958, 1959) maintained ascorbic acid at the nonchilling temperature but lost it at the chilling temperature. Lemon fruits, in contrast, lost ascorbic acid at 24°C and maintained or increased it at 4°C. However, after a chilling exposure that resulted in chilling symptoms (6 weeks at 7.5°C) the sweet potato (Lieberman *et al.*, 1959), like the lemon after 12 weeks at 4°C, showed a marked decrease in ascorbic acid at the nonchilling temperature.

Changes in ascorbic acid in the lemon fruit do not appear to be related to the mechanism or initial changes of chilling injury. The loss of ascorbic acid after a severe chilling exposure is secondary, probably associated with the symptoms that develop.

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Probable Identity of the Pectinase Inhibitor in Grape Leaves

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(Manuscript received June 26, 1961)

SUMMARY

Studies were continued on the chemical nature of the water-soluble substance in Muscadine grape leaves that was previously reported as inhibiting pectinase and cellulase. The inhibitor is a tannin or tannin-like material, removable from leaf extracts by hide powder, caffeine, gelatin, and nicotine sulfate. Muscadine leaves had the highest inhibiting activity of the plants tested.

INTRODUCTION

The importance in cucumber brining of the softening problem caused by enzymatic degradation of cell wall pectin was discussed by Etchells *et al.* (1958a, b). That paper also reported studies showing that the pectinolytic enzyme activity is chiefly the result of the growth of higher fungi (molds) in the cucumber flowers before they reach the brining station. Many of these flowers remain attached to the cucumbers when brined. Bell and Etchells (1958) and Bell *et al.* (1960) reported in Muscadine grapes (Scuppernong variety) a factor that inhibited pectinase and cellulase activity.

The grape leaf inhibitor was found by Bell and Etchells (1958) to be water-soluble, stable to heat, non-dialyzable through a cellophane membrane, and not completely precipitable by acetone or concentrated ammonium sulfate. They also found that reduction in pectinase activity was directly related to the inhibitor concentrations used and that the reaction between pectinase, substrate, and inhibitor was that of competitive inhibition.

Pectic enzymes are destroyed by the usual enzyme and protein denaturants such as heat, heavy-metal salts, strong acids, and strong alkalis. Kertesz (1951) reviewed the pectinase literature and found that compounds such as tannins, glycine, and formaldehyde were reported as being inhibitory. Later work, however, did not confirm the earlier findings on the latter two compounds. Rahman and Joslyn (1953) studied a number of the usual inhibitors such as monoiodoacetic acid, mercuric chloride, sodium azide, sodium fluoride, ammonium arsenate, and sulfur dioxide against purified polygalacturonase (pectinase) and concluded that the enzyme was generally resistant or only slightly affected by these compounds.

Naturally occurring inhibitors have been previously reported for the hydrolytic enzymes. Inhibitors for amylases were found in wheat by Militzer *et al.* (1946), and in Leoti sorghum by Kneen and Sandstedt (1946) and Miller and Kneen (1947). Weurman (1953) was the first to report a thermolabile inhibitor of pectinase, which he precipitated from pear sap with acetone.

Many investigators have demonstrated the inhibition of enzyme activity by tannins. Ehrenberg (1954) reported that the phosphatase activity of leaves of Kalanchoe was

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inhibited by the natural tannin content of the plant. The inhibition was eliminated by adding a slightly chromated hide powder to the extract. Caneghem and Spier (1955) showed that tannin, phosphotungstic acid, sodium lauryl sulfonate, thiazole yellow, and polyphloretin phosphate inhibited the action of hyaluronidase. Ishimatsu and Kibesaki (1955) showed that agarase activity was inhibited by tannic acid. Friedrich (1955) reported that the natural tannin present prevented the plant enzymes from splitting arbutin during the extraction process, and hypothesized that such protective action probably accounted for the greater medicinal value of the extract over that of crystalline arbutin. Jackson and Wood (1959) demonstrated in rose hips an inhibitor that was effective against the oxidation of ascorbic acid, and postulated that tannin may be the active principle.

Other work has shown that tannins are effective inhibitors for pectinase. Grossmann (1958) reported that pectinolytic enzymes from *Fusarium oxysporum* f. *lycopersici* were inhibited by added tannins, that chebulinic acids were less effective, and that D-catechins were ineffective. Hathaway and Seakins (1958), reporting on the inhibition of pectinase by several tannins, showed that myrabolan tannin inhibited the rate of hydrolysis but that the hydrolysis went to completion if sufficient time was allowed. This suggests a competitive type of inhibition. Gambir tannin was shown to have no effect. Cole (1956) demonstrated the inhibition of pectinase by tannin and oxidized apple juice.

Cadman (1959) showed that extraction of minced raspberry leaves with 2.5% nicotine gave a tannin-free extract that was very poor as an inhibitor in the prevention of virus infection. Barnes (1956) showed that tannin can be precipitated from water solution by N-bases such as caffeine and antipyrine.

Screening studies by the authors, on pectinase and cellulase inhibition by 61 species in 37 families of plants, have been completed and prepared for publication. The present work was initiated to obtain further information on the chemical nature of the pectinase inhibitor substance(s) in grape leaves as compared to a selected group of the screened plant samples, including those with and without inhibitory activity.

MATERIALS AND METHODS

Plant materials. Samples of grape leaves and of other plant leaves were collected at North Carolina State College and vicinity in the summer of 1960. The samples were washed with water, surface dried, and placed in polyethylene bags. All samples were stored at −15°C or below.

Extractions. Weighed samples (40.0 g) of the frozen leaves were cut with scissors and placed with 300 ml of distilled water in a Waring blender (mention of company and trade names does not imply endorsement over others not named). Toluene (1 ml) was added as a preservative, and the blender was run 30 sec at low speed and then increased to high speed for 2.5 min. The slurry was filtered through four layers of cheesecloth on a Buchner funnel and washed with 150–175 ml water. The filtrate was made to 500 ml. For viscosity measurements (inhibitory power), aliquots of this solution were centrifuged 15 min at 3000 rpm. For tannin analyses, the solutions were filtered according to the filtration procedure employed in the official hide-powder method of the American Leather Chemists Assoc. (1957).

Enzyme solution. A new enzyme solution was prepared each day. A weighed sample (0.100 g) of Pectinol 10-M (Rohm and Haas Company, Philadelphia, Pa.) was made to 100 ml with water. An aliquot of this solution was diluted to a concentration of 0.0002 g/100 ml.

Enzyme substrate. Six g of sodium polypectate (Sunkist Growers, Ontario, California) was dissolved in 500 ml of 0.02*M* NaOH-citric acid buffer at pH 5.0 and 55°C by mixing in a Waring blender. The resulting solution was filtered through several layers of cheesecloth and preserved with 1 ml of toluene. This is the solution used by Bell *et al.* (1955).

Measuring enzyme and inhibitor activity. The viscometric method of Bell *et al.* (1955) was modified by employing a more concentrated enzyme solution and by taking measurements after a reaction time of 1 hr and 2 hr instead of 20 hr. Using 100 units of pectinase activity as equivalent to a 50% viscosity loss in 2 hr of reaction time, a table was set up relating loss in viscosity to units of pectinase activity. This table was calculated from a curve relating the log of pectinase activity units to the percent loss in viscosity. Since a 10-fold enzyme concentration and only a 2-hr, instead of a 20-hr, reaction time were employed, the viscosity-activity relationships reported in the original method were the same as those calculated for this work.

Standard enzyme activity (control) was measured by mixing enzyme solution with water (2:1 v/v) and using 1 ml of this mixture added to 5 ml of substrate at 30°C in an Ostwald-Fenske viscosity pipette (uncalibrated, No. 300). Inhibitor activity was measured by substituting one volume of the inhibitor solution for the water. The enzyme and inhibitor were mixed at least 15 min before the viscosity measurement was started. Correction was made for the flow time for pure water for each pipette.

Tannin analyses. Tannin analyses were made by the official hide-powder method of the American Leather Chemists Assoc. (1957). The percent of tannin and the tannin purity values were calculated by the following formula:

$$\% \text{ tannin} = \frac{(A - B)\ 100}{C}$$

$$\text{Tannin purity} = \frac{(A - B)\ 100}{B}$$

where A = g solids per ml of extract

B = g solids per ml of extract after hide-powder treatment

C = wt sample per ml extract.

RESULTS AND DISCUSSION

Influence of concentration of grape leaf on inhibitory power of extract. Forty grams of Creek grape leaves (Muscadine group) were extracted and made to 500 ml. Serial dilutions from 80 mg/ml to 0.6 mg/ml were made, and the resulting solutions were tested for inhibitory power. The data are presented in Fig. 1. All concentrations down to and including 4.8 mg/ml gave 95% or more reduction in pectinase activity. On the basis of the tannin content (hide powder) of this sample (16.5% on a moisture-free basis) the tannin concentrations ranged from 5.2 mg/ml to 0.04 mg/ml. The concentration corresponding to 4.8 mg leaf/ml was 0.31 mg tannin/ml. As pointed out by Bell and Etchells (1958), leaves from species of grapes other than Muscadine varieties have less inhibitory activity and the inhibition starts to drop at a much higher concentration of leaf material per milliliter.

Relationship of type and quantity of tannin to inhibitory power of plant extracts. The presence of pectinase inhibitors in plants other than the grape has been demonstrated (Bell *et al.*, 1961). Several of these plants were selected to span the range of inhibitor activity from 95% to 0%, for use in a study of the relationship of tannin (as determined by hide powder) to inhibitory activity. The results are reported in Table 1. In all cases the hide-powder treatment used in the tannin analyses removed essentially all of the active principle, and removal was completed by further additions. However, the inhibitory power of the untreated extract was not perfectly correlated with its tannin content, because of three factors.

The first factor is that an increase in tannin content beyond a certain point produces little or no increase in inhibitory power (Fig. 1). An extract of Muscadine grape leaves, if diluted from 80 mg leaf/ml to 4.8 mg leaf/ml, contained 0.3 mg tannin/ml and still produced about 94% inhibition. Undiluted iris leaf extract (0.3 mg tannin/ml) and sericea extract (0.6 mg tannin/ml), each prepared from 80 mg leaf/ml, produced 88% and 80% reduction in pectinase activity, respectively. This would indicate that these three plant sources, on a tannin basis, appear to be about equaly effective. However, on a fresh leaf basis (80 mg leaf/ml) the Muscadine grape leaves appear to have 8–17 times the inhibitory capacity, because of the higher tannin content of the grape leaves.

The second factor is the varying inhibitory power of different types of tannin. This difference in activity due to tannin source is exemplified by tests on commercial tannins. Myrabolan tannin had a relatively high ac-

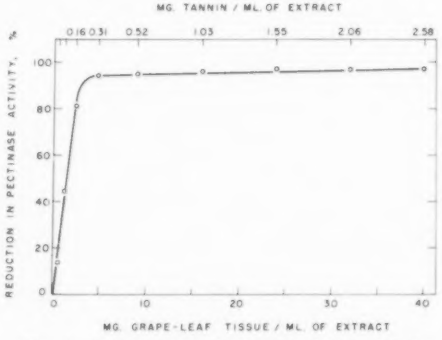

Fig. 1. Relationship of grape leaf concentration and tannin concentration to reduction in pectinase activity.

Table 1. Inhibitory activity of plants as related to tannin content.

Plant [a]		Moisture (%)	Tannin (MFB) (%)	Tannin in extract (mg/ml)	Tannin purity [b]	Inhibition of pectinase: Water extract (%)	Inhibition of pectinase: Non-tannin extract [c] (%)
(Plants with inhibitory activity)							
Muscadine Grape	*Vitis rotundifolia* var. Creek	60.0	16.5	5.3	43.7	95	7
Iris	*Iris* sp.	82.8	2.0	0.3	5.4	88	14
Sericea	*Lespedeza cuneata*	45.3	1.4	0.6	11.1	80	12
Raspberry	*Rubus strigosus* var. Latham	61.1	5.2	1.6	16.2	70	0
Strawberry	*Fragaria chiloensis*	72.6	7.4	1.6	19.6	60	0
Dogwood	*Cornus florida*	58.5	9.7	3.2	26.0	64	0
Peach	*Prunus persica*	59.7	2.9	0.9	9.4	60	0
Rose	*Rosa odorata*	61.8	3.9	1.2	14.2	57	14
Blueberry	*Vaccinium ashei*	54.3	19.3	7.1	42.6	54	21
Sweet Potato	*Impomoea batatas*	81.7	7.2	1.1	33.2	36	0
(Plants with negative to doubtful inhibitory activity)							
Tomato	*Lycopersicon esculentum*	86.3	2.6	0.3	6.7	12	3
Pear	*Pyrus communis*	55.9	6.0	2.1	19.4	11	19
Cherry	*Prunus cerasus*	63.9	2.4	0.7	8.8	0	13
Holly	*Ilex cornuta* var. Burfordii	62.1	4.1	1.2	20.2	0	4
Apple	*Pyrus malus*	53.0	4.9	1.8	12.4	0	8
Magnolia	*Magnolia grandiflora*	51.5	3.7	1.4	14.1	2	16
Fig	*Ficus carica*	68.6	4.0	1.0	11.9	0	0

[a] All samples consisted of 80 g fresh leaf tissue extracted 3 min in Waring blender at high speed with 600 ml of water, filtered through cheesecloth, and made to one liter, including washings.

[b] Tannin purity $= \frac{\%\ \text{tannin}}{\%\ \text{total solids}} \times 100$

[c] This column indicates residual inhibitory power after treatment with hide powder for tannin analysis. Further hide-powder additions produced extracts having no residual inhibition of pectinase.

tivity, and Wattle tannin had little or no activity. This confirms the work of Hathaway and Seakins (1958). It would appear that Muscadine grape, iris, and sericea may contain the same type of tannin. Blueberry, which gave only 54% reduction in activity though its extracts contained 7.1 mg tannin/ml, may be a second type of tannin. Montmorency cherry, holly, apple, and fig, which gave no reduction in activity, though their extracts contained 0.7–1.8 mg tannin/ml, probably contain tannins different from any of those above. It is apparent that a tannin with a low inhibitory efficiency would produce less enzyme activity reduction than Fig. 1 would predict, since the curve is apparently based upon a tannin of high efficiency.

The third factor, tannin purity (% tannin × 100/total solids), complicates the above considerations. Tannin extracts of low purity are known to give high tannin values by the hide-powder method, because of adsorption of non-tannins by the hide powder. Since iris and sericea gave water extracts of much lower purity than the Muscadine grape leaves, the actual tannin content may be lower than indicated by the analytical method. This would indicate that the tannin from these two sources may be more effective than the actual data show. For this and other reasons, further work is in progress on the identity of the types of tannins of high inhibitory capacity.

Influence of extractant on the inhibitory power of the extracts. A sample of Seuppernong grape leaves (Muscadine) was extracted in the usual manner with water. A second sample was extracted with 1.3% caffeine solution, and a third was extracted with 2.5% nicotine sulfate solution. The

water extract gave 95% inhibition, and the two alkaloid extracts gave no inhibition. Addition of caffeine to the water extract followed by centrifugation gave a solution producing no inhibition. Caffeine and nicotine sulfate alone had no influence on the rate of viscosity change due to the enzyme. Addition of gelatin to the leaf sample while extracting with water gave an extract having no inhibitory power.

Influence of treatment of water extracts on inhibitory power. The dialysis experiments of Bell and Etchells (1958) were repeated in more detail. An extract of Scuppernong grape leaves was prepared and diluted to a concentration of 2 mg leaf/ml. This concentration produced 80% inhibition before dialysis. The extract was dialyzed with stirring against distilled water for 2 hr, with four changes of the water outside the cellophane membrane. The dialyzed solution gave 82% inhibition, and the dialysate, after concentration *in vacuo* at room temperature to a volume equal to the starting volume, gave 7.5% inhibition. An extract containing 40 mg leaf/ml gave 98% inhibition before and after dialysis, and the dialysate gave 9% inhibition. The inhibition of the dialysate is insignificant when considered with the total inhibition. Therefore, the active inhibitor must be of relatively high molecular weight.

Adsorption on Amberlite IRA-410 (OH^-) completely removed all inhibitory materials. Elution with ammonium hydroxide and deionization with Dowex 50 (H^+) produced a dark solution that yielded a strong test for polyphenols with ferric chloride and showed an inhibitory power about 25% of that of the original extract.

A borate complex was made and adsorbed on Duolite A-4 resin charged with 0.1*M* $Na_2B_4O_7 \cdot 10\,H_2O$. All inhibitory activity was removed, and the ammonium hydroxide and sodium hydroxide eluates from the column were completely inactive. This indicates that the borate inactivates the tannin.

Using the technique employed by Bell and Etchells (1958) on grape leaf extract, we have been able to show that pectinase inhibition by myrabolan tannin is of a competitive nature.

In a preliminary gel filtration resolution with Sephadex G-50 (Pharmacia, Uppsala, Sweden), a grape leaf extract indicated 5–6 compounds, represented by elution peaks, as shown by the activity of the fractions as inhibitors of pectinase. Ferric chloride tests on the individual fractions indicate a close correlation between the polyphenol content and the inhibitory activity. Since the peaks were very quickly eluted from the column, it must be assumed that the active components were of relatively high molecular weight. The fact that multiple peaks of activity were obtained indicates a range of molecular weights.

All of the experimental work on the relation of tannin to inhibitory activity—removal of activity by hide powder, gelatin, and alkaloids; dialysis; ion-exchange studies both on natural extracts and on borated extracts; resistance to heat; and the relation of polyphenols to inhibitory power after crude separation according to molecular weight by means of gel filtration—indicates that the active inhibitor of pectinase found in Scuppernong grape leaves is a tannin or a tannin-like compound. There is always the possibility that some non-tannin compound, having many of the physical and chemical characteristics of the tannins, may be responsible, but, considering the number of positive correlations, this chance seems remote. Work is continuing on characterization of the type of tannin.

ACKNOWLEDGMENT

The authors thank Muriel L. Happich, Hides and Leather Laboratory of the Eastern Division, for making the hide-powder tannin analyses.

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Inhibition of the Denaturation by Salt of Myosin in Baltic Herring

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(Manuscript received March 18, 1961)

SUMMARY

Solubility measurements have shown that the "myosin" of Baltic herring is denatured rapidly *in situ* even in a 2% sodium chloride solution at 0°C. This denaturation is inhibited by various alkali phosphates and citrate. A higher concentration of sodium chloride requires a higher concentration of phosphate to prevent denaturation by the former. This action of alkali phosphates varies with the pH of the solution, probably as a consequence of changes in the net charge of the proteins and in the dissociation of the phosphates. Possible mechanisms of the inhibiting effect of the phosphates and citrates on the denaturation by sodium chloride are discussed.

The muscle proteins of fish may undergo denaturation from many different causes: freezing, thawing, freeze-drying, heating, various salts, etc. The mechanism need not be the same in all cases, however. Thus, Luijpen (1957) stated that the denaturation of fish proteins caused by freezing differs in principle from that caused by heating. There are also marked differences between the effects of freeze drying and heat denaturation (Hamm and Deatherage, 1960). In contrast, many factors indicate that there is an appreciable similarity between the denaturation of fish muscle proteins caused by freezing and that caused by salts. Love (1958), Simidu and Simidu (1957), Simidu and Hibiki (1951), and Luijpen (1957), among others, concluded that the denaturation of muscle protein of frozen fish is caused by the concentration of tissue salts that results from freezing.

Duerr and Dyer (1952) and Fougère (1952) stated that when cod fillets are kept in strong NaCl solutions at 0–20°C, the structural proteins are rapidly denatured when a certain "critical" salt concentration (8–10% NaCl) is reached in the muscle. However, denaturation of the proteins of Baltic herring is produced by a 2% NaCl solution, the final salt concentration in the muscle being only 1.7% (Nikkilä and Linko, 1954b). Similar results with different fish species have been obtained by Simidu and Hibiki (1951) and Simidu and Simidu (1957), who showed that denaturation occurs in 2–3% NaCl solutions, the rate of denaturation depending on the salt concentration and the size of the fish fillet.

Duerr and Dyer (1952) and Nikkilä and Linko (1954b) also showed that NaCl specifically denatures the "myosin" fraction of muscle. This denaturation of "myosin" by salt is similar to the denaturation resulting from freezing and thawing (Nikkilä and Linko, 1954a). By "myosin" is here meant that fraction extractable from the muscle with 5% NaCl but subsequently found to be insoluble in 0.25% NaCl.

Shimizu and Simidu (1953) observed with fish muscle that, for equimolar solutions the effectiveness of denaturation was in the following order: $MgCl_2 > NaCl > KCl > MgSO_4$. With Baltic herring, however, Nikkilä and Linko (1954b) found the following order of effectiveness: $NaCl > KCl > MgCl_2$, whereas glucose did not alter the solubility of myosin.

In the present study, an investigation was made of the effect of different sodium phosphates, sodium citrate, and sodium acetate on the denaturation by sodium chloride of the myosin of Baltic herring *in situ* at 0°C.

METHODS

Skinless fillets of Baltic herring *(Clupea harengus* var. *membranus)* of as uniform a size as possi-

ble were immersed in 2 or 5% sodium chloride solutions containing sodium ortho-, meta-, or polyphosphate, sodium citrate, or sodium acetate in various concentrations at 0°C and pH 6–8. In most of the experiments the concentration of sodium chloride was 2%, because the myosin of Baltic herring is denatured *in situ* rather rapidly in a sodium chloride solution of this strength. The brine was stirred occasionally during the experiments. Jars were taken at intervals for the analysis of fillets and salt solutions (*cf.* Nikkilä and Linko, 1954b).

The extractable myosin of the fish fillets was determined by a procedure described previously (Nikkilä and Linko, 1954a, b).

RESULTS

Fig. 1 shows the variation of the solubility of myosin in 5% sodium chloride solution after the fillets had been kept for different periods in solutions containing 2% (A) or 5% (B) sodium chloride and 0.5 to 3% of a phosphate mixture of 1:2 tetrasodium pyrophosphate and disodium pyrophosphate. The pH values of all the salt solutions were adjusted to 7.5 before the experiments were begun. The results show that the solubility of myosin decreases markedly in the solutions containing 2 or 5% sodium chloride (A and B, curves I), but this decrease is clearly decelerated in the presence of sodium pyrophosphate. The rate of denaturation depended on the concentration of both sodium chloride and phosphate. Thus, the concentration of phosphate mixture needed to prevent denaturation completely was 1% in the 2% sodium chloride solution (A, curve III) and 3% in the 5% sodium chloride solution (B, curve IV). Results were similar when the fillets were allowed to stand for 1, 3, and 6 days in the phosphate solutions before the addition of sodium chloride. The undenatured myosin N finally found in the sodium chloride solutions amounted to about 10% of the original protein N (*cf.* Nikkilä and Linko, 1954b).

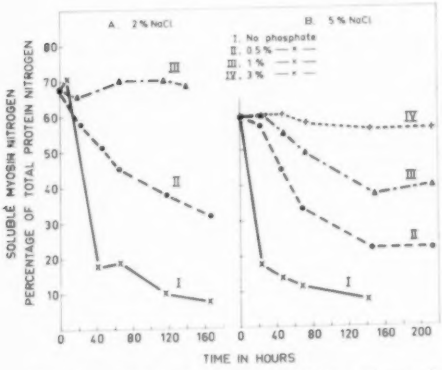

Fig. 1. The changes in the solubility of myosin from Baltic herring fillets stored in sodium chloride solutions containing added sodium pyrophosphate at pH 7.5 and 0°C.

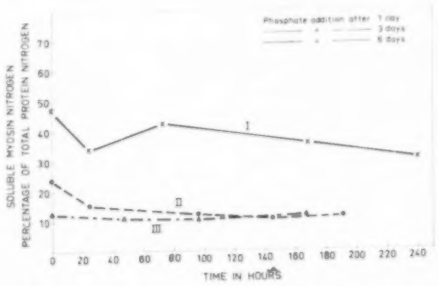

Fig. 2. The changes in the solubility of myosin after the addition (at 0 hours) of 1% sodium pyrophosphate to brine solutions of pH 7.5 in which Baltic herring fillets had been stored at 0°C. The initial myosin N percentage was 63% in all experiments.

Fig. 2 shows the variation in solubility of myosin when Baltic herring fillets were kept first for 1 (curve I), 3 (curve II), or 6 (curve III) days in a 2% NaCl solution, and an amount of the abovementioned solution of sodium pyrophosphate sufficient to raise the final concentration of the latter salt to 1% was then added to each solution. It appears that pyrophosphate is able to inhibit the denaturation of that portion of the myosin unaffected by treatment with sodium chloride but cannot reverse the denaturation already accomplished.

To check the possibility that pyrophosphate enhanced the extractability of myosin, fillets were kept in a 2% NaCl solution for 1, 3, or 6 days and then extracted in the same solutions after salt solutions had been added until they contained 5% sodium chloride or 5% sodium chloride and 1% sodium pyrophosphate.

The results (Table 1) show that addition of pyrophosphate to the extraction medium has no effect on the extractability of myosin under these conditions.

Sodium pyrophosphate alone decreases the solubility of myosin only to a small degree, as may be seen in Fig. 3 from curves I (1% of the phosphate mixture), II (3%), and III (5%). The loss in solubility reached its lowest level in the solution

Table 1. Effect of sodium pyrophosphate on extractability of "myosin."

Duration of prior treatment with 2% NaCl soln.	% soluble myosin N of total protein N	
	5% NaCl	5% NaCl + 1% $Na_4P_2O_7$
1 day	47.2	47.8
3 days	24.8	23.1
6 days	11.5	13.6

containing 5% phosphate. At the end of the experiment, after about 140 hr, the amounts of soluble myosin in the samples containing 1, 3, and 5% phosphate were respectively 65.0, 57.0, and 50.3% of the total protein of the muscle, the initial value being 69.9%.

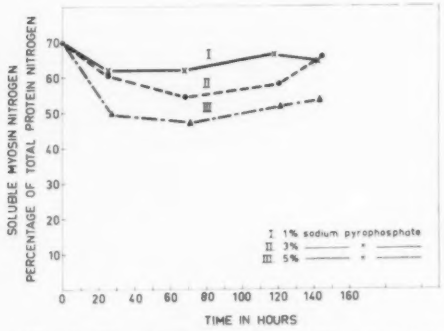

Fig. 3. The changes in the solubility of myosin from Baltic herring fillets stored in sodium pyrophosphate at pH 7.5 and 0°C.

The next series of experiments investigated the effect of different sodium phosphates on the denaturation of the fish myosin in the physiological pH range. Fig. 4 illustrates the solubility of myosin after the fillets had been kept for 210 hr at 0°C in solutions containing 2% sodium chloride (curve I), 2% sodium chloride and 2.8% sodium orthophosphate (curve II), 2% sodium chloride and 2.7% sodium pyrophosphate (curve III), 2% sodium chloride and 2.5% sodium tripolyphosphate (curve IV), and 2% sodium chloride and 2% sodium hexametaphosphate (curve V), the pH values of the solutions being adjusted with HCl or NaOH to 6, 7, or 8. The percentages vary because the amounts of phosphate salts added were such that the phosphorus contents of the solutions were equal. The figure shows that the myosin is almost completely denatured at all these pH values in the presence of sodium chloride alone (curve I), the amount of the soluble myosin being less than 10% of the total protein. All the phosphates studied inhibited the denaturation of myosin, but their efficiencies varied with pH. Sodium orthophosphate had the weakest effect at all the pH values studied, the soluble myosin varying from 30 to 36% of the total protein. Sodium pyrophosphate prevented the denaturation of myosin much more effectively than sodium tripolyphosphate or sodium hexametaphosphate at pH 6, the soluble myosin being respectively 56, 39, and 35%. At pH 7, all three were nearly equally effective, the amount of the soluble myosin varying from 55 to 57%. The effects of sodium pyrophosphate and sodium hexametaphosphate at pH 8 were the reverse of those at pH 6. The effects of the other salts were approximately the same at pH 8 as at pH 6.

Two percent trisodium citrate also prevented the denaturation of myosin caused by sodium chloride (Curve II in Fig. 5). In contrast to the citrate, addition of 1–6% sodium acetate increased the rate of denaturation, but after approx 170 hr the extent of denaturation in the solution containing sodium chloride and sodium acetate was similar to that in the solution containing only sodium chloride.

DISCUSSION

These experiments confirm that a concentration of sodium chloride as low as 2% rapidly lowers the solubility of the myosin

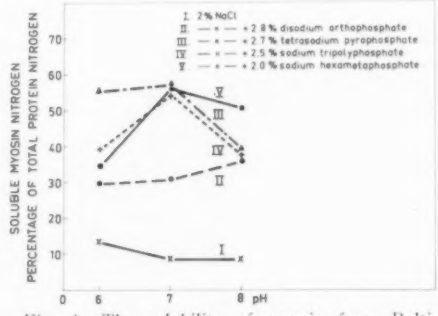

Fig. 4. The solubility of myosin from Baltic herring fillets stored for 210 hr in sodium chloride solutions containing different alkali phosphates in the pH range of 6 to 8, at 0°C.

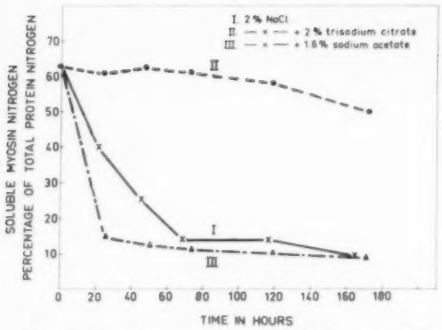
I. 2% NaCl
II — x — + 2% trisodium citrate
III — x — + 1.8% sodium acetate
SOLUBLE MYOSIN NITROGEN
PERCENTAGE OF TOTAL PROTEIN NITROGEN
TIME IN HOURS
0 20 40 60 80 100 120 140 160 180
10 20 30 40 50 60 70
I
II
III

Fig. 5. The changes in the solubility of myosin in Baltic herring fillets stored in sodium chloride solutions with the addition of sodium citrate and sodium acetate at pH 7 and 0°C.

of Baltic herring *in situ*. This decrease in solubility we have taken as the criterion of denaturation. This denaturation of myosin in sodium chloride solutions may, as our experiments show, be slowed down and even completely inhibited by small amounts of alkali phosphates in the solution. If the concentration of sodium chloride is increased, the inhibition of myosin denaturation requires a higher concentration of pyrophosphate (Fig. 1). Variations were great when the denaturation-inhibiting effect of different phosphates was studied in the physiological pH range (Fig. 4). Such variations in the action of the phosphates may be due to differences in the charges of the muscle proteins and in the dissociation of the phosphates at different pH values.

The time and order of addition of the phosphate and sodium chloride were varied in the different experiments. Phosphates inhibited denaturation if they were added to the storage solution before or together with sodium chloride (Fig. 1). If only phosphate was present (Fig. 3), no definite denaturation occurred. If the phosphate was added after the fillets had been first kept in sodium chloride solution for different periods (Fig. 2), it was able to prevent further denaturation of the myosin, but no reversal of denaturation (i.e. increase in solubility) took place. The addition of phosphate to the extraction medium did not increase the solubility of myosin. It thus seems that the phosphates directly inhibit the denaturation of myosin by sodium chloride and that the effect is not due to an increase in the solubility of myosin.

Alkali citrates also have an inhibiting effect on the denaturation of the Baltic herring myosin caused by soduim chloride. Thus, two groups of substances, phosphates and citrates, that differ greatly from each other in chemical structure, can function as denaturation inhibitors. All these substances, moreover, increase the water-binding capacity of muscle, although they do not follow the series of Hofmeister in this respect (*cf.* Hamm, 1955). In contrast, sodium acetate, which according to Hamm (1960) exerts a weak dehydrating effect in low concentrations ($\mu = 0.3$), has an opposite effect (Fig. 5).

There are various mechanisms by which phosphates and citrates could inhibit the reduction of solubility of native Baltic herring myosin by sodium chloride and at the same time increase the hydration of this protein. Phosphates and citrates may, for example, be taken up directly by protein molecules, thereby increasing the numbers of polar groups in the latter. In addition, condensed phosphates bind to actomyosin, which the myosin of this study closely resembles, increase its negative charge and cause it to dissociate. According to Bendall (1954) and Kotter and Prändl (1956), such a process leads to an increase in the solubility of this protein. Phosphates and citrates may also remove calcium and zinc ions that are bound to muscle proteins, and so increase the number of polar groups in the latter (Hamm and Grau, 1958). A similar ability to bind calcium and zinc is possessed by ethylenediaminetetraacetate (EDTA). However, in experiments performed with Baltic herring (unpublished experiments of the authors), EDTA was unable to prevent the lowering of myosin solubility by sodium chloride. The removal of calcium and zinc ions therefore may not be the correct explanation for the effects of phosphates and citrates shown in this paper.

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Influence of Irradiated Bacon Lipids on Body Growth, Incidence of Cancer, and Other Pathologic Changes in Mice[a]

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(Manuscript received March 17, 1961)

SUMMARY

Uncured bacon, irradiated with 5.58 megarads of gamma rays was compared with similar uncured unirradiated bacon by feeding to 757 mice. There were no major differences between the control and experimental groups. No gross or histopathological lesions attributable to irradiation of the lipid were observed. The incidence of spontaneous cancers showed no significant differences among the diet groups and indicates that irradiation of the bacon did not produce carcinogens or growth-altering substances demonstrable under the conditions of these experiments.

Radiation preservation of food offers the possibility of two important contributions to world food distribution: (1) extension of the shelf life of refrigerated foods; and (2) retention, without refrigeration, of most of the natural qualities of food so as to permit world-wide shipping, storage, and consumption (Siu *et al.*, 1955). Thus, types of food not now readily useful for the Armed Forces might become available.

Government approval of radiation preservation of food is required for its use. A provision of the Federal Food Additives Law bars the use of additives known to be carcinogenic or pathogenic, and it is generally accepted that induced radioactivity is an "additive" that has this potential. Therefore radiated foods need to undergo extensive tests to ascertain that no detrimental effects on growth, reproduction, and longevity occur, and that no potentially toxic or carcinogenic substances develop.

Read (1960) discussed the confusion that has arisen concerning the suitability of irradiated foods for human consumption. He also described wholesomeness testing and undertook an analysis of available data.

The work described here is part of the contractual program of the office of the Surgeon General, Department of Defense. It was undertaken to determine if gamma irradiation of bacon has any influence on the bacon lipids that would alter the incidence or rate of appearance of spontaneous neoplasms and other pathological changes in mice.

EXPERIMENTAL

Irradiation and storage. It was considered advisable to use uncured bacon because nitrites (Huber, 1957) in processed bacon might have the effect of decreasing the side reactions secondary to irradiation.

The Quartermaster Food and Container Institute for the Armed Forces obtained uncured bacon packed in 4.5-lb amounts in No. 10 cans, and shipped it to the Materials Testing Reactor, Phillips Petroleum Company, Idaho Falls, Idaho. Half of the bacon was shipped, frozen and unirradiated, to Los Angeles, where it was kept at −20° until use. The other half was retained at Idaho Falls for radiation. A month's supply of bacon for the irradiated lipid diet was gamma-irradiated (Read *et al.*, 1959) with 5.58 megarads and then shipped to Los Angeles. For the first six months of the project the irradiated bacon was kept at 5° until used, but for the remainder of the time it was

[a] The Experiments reported were undertaken under contract No. DA-49-007-MD-570 with the Office of The Surgeon General. The opinions expressed are those of the authors and are not necessarily endorsed by the Department of the Army.

stored at room temperature. In the last eleven months of the experiment, the bacon was gamma-irradiated at Savannah River, Georgia.

Animal feeding. The C3H/Crgl (formerly designated as the C3H/He Crgl (Snell *et al.*, 1960) and hereinafter referred to as C3H) and the A/Crgl (Cancer Research Genetics Lab., 1959) (formerly designated as the A/He Crgl and hereinafter referred to as A) female mice used in the experiment were obtained from the Cancer Research Genetics Laboratory (Crgl), University of California, Berkeley, California. Some sub-lines of the C3H mouse have up to a 90% incidence of spontaneous mammary cancer by the time they are 10 months old (Fawcett and Wilson, 1955), and incidence is 5–10% in the A mouse (DeOme, 1956). A comparison of the incidence of mammary cancer in the different diet groups could thus be made between the two strains of mice.

Although the C3H female mice have a less than 1% incidence of hepatoma (Barnewell, 1957), an indication of the incidence of hepatoma in females surviving past 12 months of age could also be obtained. Likewise, the occurrence of lung cancer was studied in the A mice, which have a normal incidence of 17% (DeOme, 1956).

The C3H animals were 134–147 days old when placed on the experimental diets, and the A mice were 63–152 days old. Both strains were maintained on a Laboratory Chow diet before being placed on the experiments, the C3H for 2½–3 months and the A mice for 2 weeks to 3 months. With both strains, animals of different ages were evenly distributed among the 3 diet groups.

The animals were caged in groups of 4–7 animals in 17.9 × 28.4 × 16.5-cm stainless-steel boxes with solid bottoms and screen tops. Six months after the project was started, the pine sawdust bedding was changed to 2/3 sawdust and 1/3 cedar shavings, which helped control mites on the animals. The mice were housed in an animal room maintained at approx 25.5 ± 1°.

Three hundred and ninety-four C3H and 362 A mice were employed. There were 98 C3H and 91 A mice on the basal diet, 149 C3H and 136 A mice on the unirradiated bacon lipid diet, and 147 C3H and 135 A mice on the irradiated lipid diet.

The basal diet and the diets to which the unirradiated and irradiated bacon fats were added were Ralston Purina Company Laboratory Chow. It was ground fine in a Wiley Mill. The chow contains not less than 23.0% crude protein, 4.5% crude fat, and 44.0% nitrogen-free extract and not more than 6.0% crude fiber and 9.0% ash. Hereinafter the three diet groups are coded: C = control basal diet, BUO = unirradiated lipid + ground chow, and BU6 = irradiated lipid + ground chow.

After six months, the lipid content of the diet was increased from 10% to 15%, and after an additional three months to 20%. For the lipid diets, the irradiated and unirradiated bacon was sliced and then fried in the regular manner until thoroughly cooked. The bacon was fried at weekly intervals. The lipid was strained through a fine metal sieve into cans, which were kept at 5° until needed. The lipid was weighed and melted over low heat before it was added to the ground Purina Laboratory Chow. Quantities of diet were mixed in a Hobart Mixer for one week's supply, and then stored in ice cream cartons at 5° until fed.

The animals were fed daily *ad libitum*, and no attempt was made to record food intake. Mice on all three diets ate their food readily and appeared to find the bacon lipid diets palatable.

During the entire experiment, cages were checked daily for deaths. During the first 2½ months of the diet, the animals were examined and weighed by cage groups twice a week. For the remainder of the experiment the animals were weighed once a week. Animals with palpable neoplasms or other gross abnormalities were weighed separately and inspected daily.

Chemical studies on lipids. Unsaturation of fatty acids was determined by the Wij's iodine titration method (Gunstone, 1958) on each can of bacon lipid. The values are expressed as the ml of 0.2*N* iodine absorbed per gram of lipid.

The peroxide number was determined by a slight modification of the method of Polister and Mead (1954). The peroxide numbers were obtained by multiplying by 1000 the ml of 0.01*N* sodium thiosulfate used per gram of lipid.

RESULTS

Growth and weights. Table 1 shows the average animal weight in grams by diet group at different intervals during the experiment. In view of the relatively high percent lipid in the unirradiated and irradiated diets as compared to the approximately 4.5% crude fat in the basal die, it would be expected that the animals on the lipid diets would weigh more. Although the control-diet animals of both strains show a consistently lower average weight, these lower weights were only 0.1–0.5 below the standard deviation of the three diet groups.

Mortality and life span. The life span of all C3H and A animals on the three diets is shown on Figs. 1 and 2. Animals were sacrificed when they showed clinical symptoms of illness or appeared to be terminal from their carcinomas. Although it was realized that the life span of animals so sacrificed may have been shortened artificially, these animals were included with those found dead in the life span data in Figs. 1 and 2.

Table 1. Average weight (g) of C3H and A strain mice fed control unirradiated lipid, and irradiated lipid diets.[a]

Months on diet	C3H				A			
	Control (C)	Unirrad. lipid (BUO)	Irrad. lipid (BU6)	Std. dev. among diets	Control (C)	Unirrad. lipid (BUO)	Irrad. lipid (BU6)	Std. dev. among diets
0	24.8 (98)[a]	25.3 (149)	25.0 (147)	± 0.25	20.1 (91)	20.6 (136)	19.9 (136)	± 0.36
3	32.8 (98)	38.5 (149)	36.5 (146)	± 2.89	25.5 (89)	28.4 (132)	27.3 (135)	± 1.46
6[b]	35.9 (86)	43.3 (137)	41.0 (128)	± 3.79	26.9 (88)	29.8 (127)	28.5 (131)	± 1.45
9[c]	37.7 (64)	47.0 (92)	44.3 (106)	± 4.78	27.1 (84)	30.0 (116)	28.1 (122)	± 1.47
12	35.9 (48)	46.0 (48)	43.0 (59)	± 5.20	25.7 (65)	25.9 (83)	24.5 (85)	± 0.76
15	37.0 (23)	44.7 (29)	40.4 (43)	± 3.86	24.7 (31)	26.4 (22)	23.8 (29)	± 1.32
18	33.6 (14)	39.7 (18)	37.3 (22)	± 3.07	24.1 (14)	27.3 (2)	22.5 (2)	± 3.42

[a] Number of animals in parentheses.
[b] Fat % was increased from 10 to 15 when animals had been on diet 6 months.
[c] Fat % was increased to 20 when animals had been on diet 9 months.

Two hundred and five of the 305 C3H mice that developed mammary cancer were 265–457 days old when tumors were first noted. Average age at time of sacrifice or death of tumor-bearing C3H mice was 480 days in the C group, 463 days in the BUO group, and 507 days in the BU6 group. For the group of 89 C3H mice that did not develop mammary cancer, average age in days at the time of sacrifice or death was 624 for the C diet, 623 for the BUO, and 650 for the BU6. Animals having hepatomas were sacrificed at average ages of 635 days for the C group, 639 for the BUO and 692 for the BU6.

No mammary-gland cancers were found in A mice on the C diet. In the BUO group the 2 animals with mammary cancer were sacrificed at an average age of 383 days. In the BU6 group the average age at the time of sacrifice for three animals was 433 days. Average age at the time of sacrifice or death for A mice with lung cancers was 540 days on the C diet, 570 days on the BUO, and 539 on the BU6.

Neoplasms. The mice studied for pathological changes totaled 744. Clinically ill or terminal tumor-bearing animals were sacrificed by ether anaesthesia, and autopsies were performed immediately. Animals found dead were autopsied as soon as possible. Tissue sections were taken of all neoplasms and of other gross abnormalities by the pathologist. Tissues were fixed in 10% formalin. Paraffin sections were stained with hematoxylin-eosin.

In these experiments the C3H mice had mammary cancer incidences of 75.5% in the C diet group, 81.2% in the BUO diet group, and 75.3% in the BU6 diet group. Approx 45% of the C3H mice with mammary tumors developed 2–8 separate tumors. The tumors were weighed at the time the animals were autopsied. Table 2 indicates the average tumor weight in each diet group; the mean of the average weights in each diet group were 8.18 g, with a standard deviation of ±0.23 g.

An attempt was made to find an estimate of the rate of mammary tumor growth in each diet group. The tumors were measured when first noted, each time the animals were weighed, and finally when the animal was sacrificed. Tumor size was obtained by multiplying the three dimensions of the tumor in centimeters. The "average rate of growth of mammary tumors" was taken as the difference between the initial and final "cubic centimeters" (cc) of each tumor divided by the number of days the tumor grew. The average values obtained by this method were 0.291 cc per day for the C group, 0.282 cc per day for the BUO, and 0.275 cc per day for the BU6. There was a wide range of "rates of

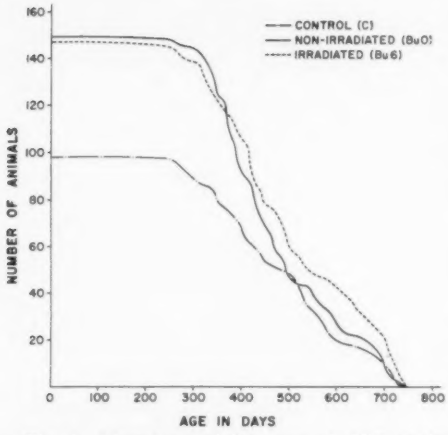

Fig. 1. Mortality and life span of C3H strain mice on C, BUO, and BU6 diets.

growth" within each diet group, and also in the "rate of growth" of several tumors in the same animal. Ranges recorded in the diet groups were: 0.0184–09460 cc per day in the C, 0.0036–1.2455 cc per day in the BUO, and 0.0017–1.9568 cc per day in the BU6.

Pulmonary metastases were found in 80 of the 305 C3H mice with mammary cancer. Metastases were present in 27.8% of the C, 25.6% of the BUO, and 26.4% of the BU6 mice with mammary tumors.

Hepatomas were observed in 20 of the 392 C3H mice that were autopsied, with an incidence of 5.2% in the C group, 6.0% in the BUO group, and 4.1% in the BU6 group. The Berkeley strain of C3H mice has been observed to have less than 1% incidence of hepatoma (Barnewell, 1957). Although the incidence of hepatoma is higher in this experiment, there appears to be no significant difference among the three diet groups ($P > 0.50$, chi-square test).

The Berkeley strain of A mice has a 5–10% incidence of mammary cancer and a 17% incidence of lung cancer (DeOme, 1956). In this experiment, no mice on the C diet developed mammary cancer whereas 2 mice (1.4%) in the BUO group and 3 (2.2%) in the BU6 group developed mammary cancer. Observed incidence of lung cancer was 3.4% in the C group, 6.1% in the BUO, and 3.8% in the BU6 ($P > 0.50$).

Table 3 summarizes the incidence of benign and malignant neoplasms in C3H and A mice in the three diet groups.

Non-neoplastic pathological changes. Table 4 records the incidences of the most common non-neoplastic pathological findings observed in this experiment. Many other non-neoplastic pathological changes were observed as single cases among the 3 diet groups, but are omitted because of the lack of significant numbers. Infections noted most frequently in A mice were interstitial disease and cystic change in the kidneys ($P > 0.50$) and granulomatous abscesses in the liver ($P > 0.50$). Salpingitis was often found in the uterine horns of both A ($P > 0.50$) and C3H mice ($P > 0.50$). As indicated by the P values, there was no significant difference in the occurrence of these infections among the diet groups.

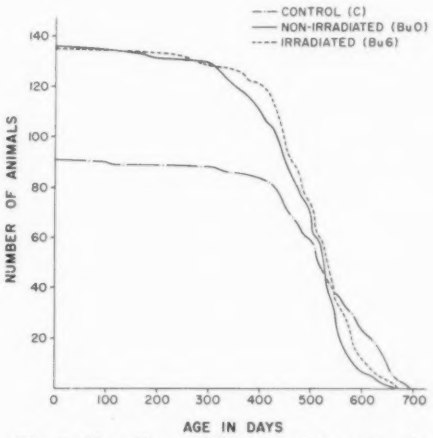

Fig. 2. Mortality and life span of A strain mice on C, BUO, and BU6 diets.

Table 2. Average weight (g) of mammary neoplasms in C3H and A mice fed control, unirradiated lipid, and irradiated lipid diets.

	C3H			A		
Diet code	No. animals with neoplasms	Range of wt/neoplasm	Av neoplasm wt	No. animals with neoplasms	Range of wt/neoplasm	Av neoplasm wt
C	74	0.1–25.4	7.9	0	0	0
BUO	121	0.2–31.9	8.2	2	3.4–4.4	3.9
BU6	110	0.5–24.3	8.5	3	2.8–7.0	5.1

Table 3. Summary of neoplasms in C3H and A strain mice fed control, unirradiated lipid, and irradiated lipid diets.

	C3H			A		
	Control (C)	Unirrad. lipid (BUO)	Irrad. lipid (BU6)	Control (C)	Unirrad. lipid (BUO)	Irrad. lipid (BU6)
No. animals beginning of diet	98	149	147	91	136	135
No. animals autopsied	96	149	147	88	131	133
Neoplasms (Malignant)						
Adenocarcinoma						
1. Lung	0	3	1	3	8	5
2. Mammary gland	74	121	110	0	2	3
3. Ovary	2	0	0	0	0	0
4. Other organs	0	0	1	1	1	0
Carcinoma-squamous cell	0	0	1	1	0	0
Carcinoma in lmyph nodes unknown origin	0	1	0	0	0	0
Hepatoma	5	9	6	0	2	0
Lymphoblastic Leukemia	2	1	3	6	6	3
Sarcoma	1	1	1	0	1	0
Thymoma	0	0	0	0	0	1
Neoplasms (Benign)						
Bronchial adenomas	0	0	4	23	21	23
Cysts						
1. Ear	0	0	0	1	1	0
2. Liver	0	0	1	0	0	0
3. Mesentary	0	0	1	1	0	0
4. Ovary	9	10	20	3	6	9
5. Skin	0	1	0	0	0	0
Hemangioma	0	0	1	0	0	0
Lymphangioma	0	0	0	0	1	0
Squamous papillomas	0	0	0	0	1	2
Total neoplasms						
Malignant	84	136	123	11	20	12
Benign	9	11	27	28	30	34

A similar cystic change in kidneys was reported by Gorer (1940) in about 100% of Strain A mice more than 487 days old. The A mice in this experiment having cystic kidneys when autopsied averaged 562 days old in the C group, 520 days in the BUO group, and 522 days in the BU6 group. Strain A mice without evidence of this renal disease when sacrificed averaged 433 days old on the C diet, and 410 on both the BUO and BU6 diets.

Dunn (1944) found a high incidence of renal disease in A strain mice, but found only one case in 150 C3H autopsies. In this experiment renal disease was observed in one C3H mouse on the C diet, in 2 on the BUO diet, and in none on the BU6 diet.

Chemical studies of lipids. Unsaturation of fatty acids and peroxide number were determined on each can of bacon lipid.

Wij's iodine method for unsaturation gave average values of 49.7 for the BUO lipid and 50.1 for the BU6. With BUO lipid, the unsaturation values ranged from 46.5 to 54.1 among the 53 cans, with a ± 1.6 standard deviation. The unsaturation values for the BU6 lipid ranged from 46.2 to 54.0 among the 56 cans, with a ± 1.5 standard deviation.

The average peroxide number of the BUO bacon lipid was 7.9, and of the BU6 lipid 12.1. The peroxide numbers ranged from 2.9 to 17.0 for the BUO lipid, with a ± 3.0 standard deviation. The range of peroxide numbers for the BU6 lipid was 5.8–30.6, with a ± 4.6 standard deviation.

Before the storage temperature of the irradiated bacon was changed from 5° to room temperature, an experiment was conducted to see if the higher storage temperature would affect the peroxide number of the lipid. Cans of BU6 bacon were stored at room temperature and opened after 2 and 4 months of storage. The bacon was fried and the peroxide number of the lipid determined. The peroxide number was 9.8 after 2 months and 15.3 after 4 months of storage at room tempera-

ture. These values fall within the range of values obtained for the 56 cans of BU6 bacon lipid.

In another experiment cans of BUO and BU6 bacon were fried, the lipid was stored at room temperature, and samples were taken periodically for peroxide determination. The results are shown in Table 5.

DISCUSSION

Rubin and Metlitsky (1958) reported that the USSR Central Inspectorate of Public Health, on the basis of tests carried out while feeding potatoes treated with 10,000 and 50,000 r to two generations of rats and dogs, has authorized the use of potatoes irradiated with a dose of 10,000 r as food in Russia.

The research of Teply and Kline (1956) failed to demonstrate the production of carcinogens on radiation of brain and egg preparations, a milk-beef-pork-fish-cheese-mixture, beef and yeast sterol concentrate mixture, peanut-corn-cottonseed oil emulsion and liquid lard. The materials were fed, injected, and painted, employing mice as the experimental animal.

Poling *et al.* (1955) observed in rats no gross or histopathological lesions attributable to a radiated beef diet. The small and occasionally statistically significant decreases in growth, food efficiency, reproduction, adult body size, and survival were considered to be due to slightly decreased nutritional quality similar to that which occurs during heat sterilization—not an indication of toxic effects.

Research of Burns *et al.* (1960) on feeding gamma-irradiated potatoes to rats demonstrated no effects on growth, reproduction, hematology, or pathologic changes.

Coleby (1959) described some of the complexities involved in determining the chemical changes produced in lipids by irradiation. In general each fat, when irradiated, behaves in a characteristic fashion, with vegetable oils often more resistant to changes than animal fats—presumably because of the higher content of natural antioxidants in vegetable oils.

Morgan (1958) has included, as reactions in irradiated lipid material, hydrogen abstraction, addition of H^+ and OH^- to double bonds, isomerization, hydrolysis, polymerization, and decarboxylation.

Our results indicate no significant differences between the degree of unsaturation and peroxide numbers of the cans of lipid

Table 4. Summary of most common non-neoplastic pathological changes in C3H and A strain mice fed control, unirradiated lipid, and irradiated lipid diets.

	C3H			A		
	Control (C)	Unirrad. lipid (BUO)	Irrad. lipid (BU6)	Control (C)	Unirrad. lipid (BUO)	Irrad. lipid (BU6)
No. animals autopsied	96	149	147	88	131	133
Kidney						
Hydronephrosis	0	0	0	5	9	7
Interstitial disease: cystic change	1	2	0	70	91	105
Liver						
Fatty changes	3	12	5	5	5	5
Granulomatous abscess	0	0	0	11	11	17
Lung						
Atelectasis	2	0	2	10	9	9
Pulmonary edema	1	1	5	6	11	19
Mesentery & mesenteric vessels						
Infarction with fat necrosis, inflammation and fibrosis	1	12	8	0	0	0
Mesenteric lymph nodes						
Dilatation of sinuses with hemorrhage and edema	6	2	4	0	0	0
Uterine horn						
Salpingitis	29	39	44	14	17	15

Table 5. Effect of room-temperature storage on lipid peroxide number.

Time after frying	Peroxide number	
	BUO	BU6
2 hours	10.9	8.1
24 hours	12.1	9.6
2 weeks	15.7	29.6
2 months	167.6	231.8

used in the diets. Storage of the lipid material at room temperature for up to two months resulted in a higher peroxide number for the irradiated lipid.

Important variables to be controlled because of their effects on sensitivity to radiation damage of food lipids are such factors as rate and amount of radiation dose, oxygen presence during irradiation, and length and temperature of storage.

It is clearly evident that the three diets in this experiment supported a good rate of growth for both strains of mice. The tendency for animals of the unirradiated and irradiated lipid diets to gain more weight than those on the control diet is believed to be due to the additional 10–20% lipid in these diets, which were fed *ad libitum*. Control and experimental animals did not differ significantly.

The incidence of benign and malignant tumors was quite similar for both groups, indicating that irradiation of the bacon did not produce carcinogens demonstrable under the conditions of these experiments.

Other histological abnormalities observed showed no significant differences among the three diet groups. It is possible that the high incidence of renal disease and early death in A mice contributed to an incidence of adenocarcinomas of the mammary gland lower than previously observed (DeOme, 1956).

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Bacteriological and Serological Studies of Organisms of the Arizona Group Associated with a Food-Borne Outbreak of Gastroenteritis[a]

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(Manuscript received June 26, 1961)

SUMMARY

The biochemical and antigenic characteristics of a strain of the *Arizona* group (1, 2:1, 2, 5) that appears to have been responsible for the illness of 51 nurses in a food-borne outbreak of severe gastroenteritis in an Institution in Washington are described. A custard prepared from fresh, unbroken eggs, was probably the vehicle of the infecting organism. The agglutination reactions of the blood serum of exposed individuals with antigens of several Enterobacteriaceae, including the *Arizona* strain isolated during the outbreak, are recounted.

INTRODUCTION

A food-borne outbreak of severe gastroenterjtis (Verder *et al.*, 1946; Vivino *et al.*, 1947; Murphy and Morris, 1950) occurred in a group of 158 nurses in an Institution in Washington, D. C., in August, 1946. Fifty-one nurses became acutely ill within 12–24 hours of eating a custard containing organisms of the *Arizona* group. It is not known how many of the 158 nurses had the custard; it was served at lunch and again with the evening meal to some of the girls, but since it was a Saturday many did not have either meal in the dining room.

The more severely ill patients were hospitalized for more than a week; in some the fever persisted many days. Eight of the more severely ill patients, who had shown no response to 3 days of therapy with sulfonamides and penicillin, were given streptomycin both intramuscularly and orally. Organisms of the *Arizona* group were present in the stool cultures of all eight before therapy was begun. In 3 patients the temperature fell to normal rapidly, in 3 a drop in temperature was dramatically rapid and in the other 2 a less abrupt drop occurred. The type-specific *Arizona* strain was still present in the stools of 4 of these patients 6 days after completion of the streptomycin therapy (Vivino *et al.*, 1947). Two months later, when 18 of the girls were questioned about recovery from their illness, 15 complained of their hair falling out and 10 reported the persistence of loose stools with cramping. A prolonged period of malaise followed the acute illness in most patients (Verder *et al.*, 1946).

METHODS

Specimens. Since the National Institutes of Health was invited to assist with the investigation of the outbreak more than 48 hours after the onset of illness in most of the patients, there was no opportunity to examine specimens before therapy was instituted.

Stool specimens from 22 patients were examined; single specimens only were available for 19, and 2 from each of 3 other patients. Fortunately, the Laboratory of the District of Columbia Health Department had isolated the organism from the stools of an additional 5 patients. No specimens were obtained from the other 24 patients. However, 16 nurses who were not ill supplied us with stools for study, and the cook, who suffered a mild illness, contributed a stool for examination.

Blood samples were secured from 9 acutely ill girls; the serum was separated from the clots for use in the serological work and the clots were cultured. Eight urine samples from hospitalized patients were cultured.

[a] From the U. S. Department of Health, Education, and Welfare, U. S. Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Bacterial Diseases, Bethesda 14, Maryland.

Blood serum was available for 42 nurses: 32 who had been ill and 10 who did not become ill though living in the nurses' residence at the time of the outbreak. Multiple samples were available for 17 patients; they were obtained during the acute illness and/or 6, 14, or 26 weeks after the clinical onset. Only eight samples were collected from the nurses who had not been ill; 7 were obtained 14 weeks after the contaminated custard was served, and three 26 weeks afterward. Since many of the nurses had transferred to other institutions immediately after their recovery, it was difficult to procure convalescent serums.

Bacteriology. Four plates of different media and 2 enrichment media were inoculated with each stool specimen: one plate each of bismuth sulphite medium (modified Difco), MacConkey's medium, SS agar, and 5% rabbit blood agar; and 2 tubes each of Selenite F (BBL) and tetrathionate (modified Difco) enrichment broth. Only the enrichment media were used with urine specimens. Heart infusion broth containing para-amino-benzoic acid was selected for the blood cultures. Every effort was made to culture the material within a few hours of collection of each specimen.

All colonies with any resemblance to those of enteric pathogens were selected for further study. Both the biochemical characteristics and the antigenic components of all cultures with properties that indicated a similarity to recognized pathogens were examined.

Biochemical methods. In an attempt to distinguish the group or division to which the selected cultures belonged, tests were made for production of indol, hydrogen sulfide, acetyl-methyl carbinol, and urease; for utilization of sodium citrate; and for fermentation of dextrose, lactose, and sucrose. When it appeared that organisms resembling *Salmonella* strains were being isolated, the fermentation of additional sugars and the liquefaction of gelatin were studied in an effort to identify the strains more closely with the *Salmonella* or with related groups (Ewing and Edwards, 1960; I.A.M., 1954, 1958).

Antigenic analysis of isolated strains. As soon as the biochemical studies indicated that the isolates had many of the properties of *Salmonella*, slide agglutinations with pooled *Salmonella* O serums were included for preliminary screening of living cultures. Tube tests with pooled *Salmonella* H serums and formalinized broth cultures were employed for examination of flagellar components. When it appeared that the O components were related to those of *S. poona* (13,22:z:1,6) and *S. worthington* (1,13,23:1,w:z), and the H components to those of *S. cerro* ($18:z_4,z_{23}$), these specific *Salmonella* antiserums were utilized to identify other freshly isolated strains. Recriprocal absorption studies were needed to clarify the serological findings because of the close relationships between both the H and O antigens of some of the *Salmonella* and *Arizona* strains. Two of the strains, freshly isolated from patients of the outbreak, were used for the preparation of specific H and O antiserums by the methods described by Edwards and Ewing (1955) for *Salmonella* antiserums. When both the biochemical and the antigenic characteristics of the isolated cultures proved atypical for previously described strains of true *Salmonella*, cultures of 6 of the isolates and the specific H and O antiserums were forwarded to Dr. P. R. Edwards for further study.

Agglutinins in patients' serums. Each serum was tested for agglutinins for each of 6 antigens: O antigen of an "outbreak" strain, H antigen of the same "outbreak" strain, O antigen of *S. poona*, and O antigens of *S. typhi*, *S. paratyphi* A, and *S. paratyphi* B. In this way the rise in the specific agglutinins of the infecting organism could be compared with those of an organism with the same minor O agglutinogens (*S. poona*) and also with the agglutinins previously stimulated as a result of T-A-B vaccination. The O antigens consisted of living organisms washed from agar slants and suspended in 0.85% NaCl. The H antigens were actively motile organisms grown in broth and killed in formalin: a 16-hr broth culture was diluted with an equal amount of 0.85% NaCl solution containing 0.6% formalin. All tests were incubated at 50°C; those with O antigens for 18 hr and those with H antigens for 2 hr.

RESULTS

A strain of the *Arizona* group (1,2:1,2,5) was isolated from the stools of 18 (67%) of 27 nurses from whom specimens were available; specimens were not obtained from the other 24 who were ill. None was found in the stools of 16 nurses who were not ill. Eight urine specimens and 9 blood clots were cultured, but none was positive. The same strain was isolated from the custard that was probably the vehicle that spread the infection; it was also isolated from the stools of the cook, who ate some of the custard for her lunch and suffered a mild illness. (The Laboratory of the District of Columbia Health Department kindly forwarded to us 25 strains isolated early in the outbreak, including the culture from the custard and also cultures from stools of 5 nurses from whom we did not obtain specimens.)

All of the strains had the biochemical characteristics of typical cultures of the *Arizona* group (Edwards *et al.*, 1959). All produced acid and gas in the fermentation of arabinose, dextrin, dextrose, galactose, glycerol, levulose, maltose, mannitol, rhamnose, sorbitol, trehalose, and xylose. All

failed to ferment adonitol, amygdaline, dulcitol, erythritol, inositol, inulin, raffinose, salicin, and sucrose in 21 days. The length of time required for detection of the fermentation of lactose varied: 2 strains produced a slight acidity in 6 days, 12 showed definite fermentation in 14 days, and the remainder gave no evidence of splitting the sugar in broth in 21 days. However, a very slight acidity was usually noticeable in most cultures in brom cresol purple milk within 10–14 days. Gelatin (12%) was liquefied in 16–21 days when incubated at room temperature in stoppered tubes. Hydrogen sulphide was produced in abundance, urea in Christensen's medium was not hydrolyzed, and indol was not formed. The methyl-red reaction was positive, the Voges-Proskauer test negative. A very slight acidity developed in Jordan's tartrate medium.

The mouse pathogenicity of 2 freshly isolated *Arizona* strains was tested by injecting intraperitoneally 0.5 ml of mucin suspensions of varying densities. Between 150 and 200 organisms of each culture killed 8 of the 10 inoculated mice within 36 hours. A strain of *S. poona,* studied similarly, proved to be equally pathogenic. A strain of *S. cerro* failed to kill unless 500 million organisms were given.

Antigenic analysis of strains isolated. All of the strains isolated during the outbreak were agglutinated by each of the specific H and O antiserums prepared for 2 cultures obtained from patients.

The isolated strains were agglutinated also by *S. poona* (13, 22: z: 1, 6) O serum and by *S. cerro* (18: z_4, z_{23}) H serum. These reactions were helpful in selecting cultures for further study during the early work on the outbreak. However, when specific O serums for the strains associated with the outbreak were available for recriprocal absorption agglutination tests, *S. poona* did not absorb the major agglutinins from the specific O serum; the 2 strains possessed only similar minor agglutinogens. In contrast, the major antigenic factors of the flagella of the freshly isolated strains and of *S. cerro* were identical; cultures of each of the strains completely removed the agglutinins for both strains from both antiserums.

The 6 cultures forwarded to Dr. P. R. Edwards were identified as strains of the *Arizona* group of the same serological type as the one originally isolated by Caldwell and Ryerson (1939), with the antigenic formula 1, 2: 1, 2, 5 (Edwards and West, 1959).

Agglutinins in blood of patients and contacts. Table 1 shows the changes that occurred in the

Table 1. Results of agglutination tests on serums of blood taken during acute illness and convalescence of 17 nurses.

		Reciprocals of titers of agglutinins in serums obtained at following times for various O and H antigens											
		Acute illness		6 weeks later				14 weeks later				26 weeks later	
	Arizona organism isolated from stool's	Arizona		Arizona		S. Poona	S. Typ. A & B	Arizona		S. Poona	S. Typ. A & B	Arizona	
Patient		O	H	O	H	O	O	O	H	O	O	O	H
1	+	0	0	640	320	40	40	320	80	—	40	0	0
2	+	20	20	160	80	20	40	160	160	—	20	0	0
3	+	40	40	640	320	40	20	0	0	0	0	0	0
4	+	0	0	80	320	40	20	160	160	20	20	40	160
5	+	40	40	320	160	20	80	320	80	20	80	0	0
6	—	0	0	0	0	0	0	160	40	—	20	20	20
7	+	160	80	1280	640	80	40	0	0	0	0	80	160
8	+	0	0	640	320	320	40	160	80	—	40	0	0
9	+	0	0	640	160	40	20	80	40	—	40	0	0
10	+	0	0	640	320	40	20	160	160	20	20	0	0
11	0	80	40	640	320	160	160	0	0	0	0	0	0
12	—	80	80	0	0	0	0	320	80	20	20	0	0
13	0	80	320	1280	640	40	160	160	320	20	40	0	0
14	+	0	0	320	320	40	20	80	80	—	40	80	40
15	+	0	0	320	160	20	20	160	160	—	20	0	0
16	+	0	0	0	0	0	0	160	160	20	20	—	80
17	+	40	40	80	80	20	80	80	160	—	80	0	0

S. Typ. A & B-O, O antigens of *S. typhi*, *S. paratyphi* A, and *S. paratyphi* B. Titer given is highest one obtained with any one of 3 antigens.

0, serum not available.

—, Titer $<1:20$.

specific agglutinin titers in the blood serum of some of the nurses during the weeks following their acute illness. The reactions with several antigens are included in order to demonstrate various types of antibody response—specific and nonspecific. The titers obtained with the O antigens of *S. typhi*, *S. paratyphi* A, and *S. paratyphi* B show the response to antigens previously received in a vaccine. The very slight rises in antibodies combining with the O antigen of *S. poona* indicated the reactions to an organism antigenically related through minor agglutinogens to the *Arizona* strain. The much higher and rising titers of specific agglutinins for both the H and O antigens of an isolate obtained from an ill nurse point toward the strain of the *Arizona* group as the etiological agent.

At least one serum sample of 25 of the 32 ill nurses (78%) from whom blood was obtained showed a titer of 1:80 or greater for both H and O antigens of the *Arizona* strain. Only 2 of the 10 nurses who did not become ill and had blood examined, possessed specific agglutinins in these dilutions; it is possible that they had had mild unrecognized infections.

DISCUSSION

This outbreak is the first mass food infection in which a completely identified *Arizona* type was recognized. Although Edwards has now studied *Arizona* strains from more than 159 outbreaks (Edwards *et al.*, 1956a, b, 1959), the laboratory findings accumulated during investigation of this outbreak are somewhat unique.

The vehicle by which the organisms of the *Arizona* group were spread appears to have been a custard prepared with fresh, unbroken eggs. Although the type-specific *Arizona* strain was isolated from the stools of the cook, it is very unlikely that she contaminated it. Since she experienced a mild diarrheal illness after eating the custard, it is not probable that she was a carrier of the agent responsible for the outbreak. It was an explosive outbreak and there is no reason to suspect that earlier scattered infections had occurred in the group. It is much more probable that the eggs either contained large numbers of the organisms or their shells were heavily contaminated, and the custard proved to be an excellent medium for their multiplication since it stood in a warm kitchen on an August day awaiting additional refrigeration. The eggs had been obtained directly from a warehouse. It was learned that the eggs delivered to the Institution had been taken from several hundred dozen picked up from various farming areas, so no further effort was made to locate eggs for study. Of the 2634 cultures of this group that Edwards (1959) and his co-workers have typed, 136 were obtained from eggs or egg products.

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Evidence for Heat Injury in Enteroccocci[a]

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(Manuscript received May 10, 1961)

SUMMARY

The death rates of stationary-phase brain heart infusion-broth cultures of *S. faecium* and *S. faecalis* were independent of nitrite at 148.5±1.5°F, but were accelerated at 155±1.5°F and 158.5±1.5°F. Ascorbic acid added to the culture at the time of heating reversed the nitrite effect. Evidence was obtained for thermal injury of enterococci surviving thermal processing in a cured-meat product. One half of the pack received a slightly longer process at 159°F than did the other. The enterococci surviving the lesser schedule eventually multiplied in the product, whereas those still viable after the lengthier process died out during storage at 50°F.

Nitrite has been described as the ingredient primarily responsible for the stability of lightly processed unrefrigerated cured meats (Silliker *et al.*, 1958). The authors presented evidence that the level of nitrite commonly used in cured meat products (78–156 ppm), in combination with mild thermal processing, rendered the surviving bacterial spores incapable of growth in the product, although subculture of the meat in appropriate media resulted in outgrowth of surviving spores.

Nitrite also appears to be important in the stability of perishable cured meats, in which thermoduric cocci are frequently a spoilage problem. *Streptococcus faecium* has been implicated as the lactic acid organism most likely to survive the processing applied to perishable canned hams and luncheon meats (Barnes and Ingram, 1955; Ingram and Barnes, 1955; Drake *et al.*, 1960).

The present studies were concerned with the response of *S. faecium* and *S. faecalis* to heat in the presence of nitrite. The results indicate that these organisms, under certain conditions, show a heat-injury phenomenon similar to that observed in spore-forming bacteria.

MATERIALS AND METHODS

Cultures of *S. faecium* HS-5 and *S. faecalis* 3 GAP-2 were obtained from the American Meat Institute Foundation culture collection through the courtesy of Dr. C. F. Niven, Jr. Eighteen-hour cultures grown at 98°F in tubes containing 5 ml brain heart infusion broth (Difco) were stored frozen until used as inocula for 100 ml of brain heart infusion broth in screw-capped 8-oz bottles. The 100-ml cultures were incubated 24 hr at 98°F, at which time the population had leveled off at 1–2 billion cells per ml. The pH of these cultures was 6.3–6.4.

Sodium nitrite and ascorbic acid solutions were prepared fresh on the day of use, and Seitz filtered. These reagents were added aseptically to the culture bottles at the beginning of each experiment. Thermal death studies were carried out by heating the culture bottles in a constant-temperature circulating water bath. Samples were withdrawn at intervals and plated in tryptone yeast extract agar (Difco). A water-blank bottle fitted with a thermometer permitted following the temperature of the culture bottles throughout the experiment. The tryptone yeast extract agar plates were incubated 48 hr at 98°F before being counted.

The test product used in the inoculated pack tests was prepared as follows: 35 lb of pork was ground through a ½-in plate and mixed with the following materials: 550 g NaCl, dextrose, 320 g corn-syrup solids, 7 g white pepper, 7 g coriander, 7 g $NaNO_2$, 7 g $NaNO_3$, and 450 g water. One hundred grams each of 24-hour cultures of *S. faecium* and *S. faecalis* were then added, and the emulsion was vacuum-mixed for 5 min. This was followed by regrinding the product through a 3/16-in plate and vacuum mixing for an additional 5 min. Six-ounce (300 × 200) cans were hand filled and sealed under 20 in. of vacuum. One half of the cans were processed 69 min in an open kettle at 159°F. A maximum center temperature of 156.5°F was

[a] Presented at the Twenty-first Annual Meeting of the Institute of Food Technologists, New York, May 8, 1961.

Table 1. Effect of nitrite or survival of stationary phase *Streptococcus faecalis* cultures subjected to heat.

Minutes in temperature range before sampling	Log *S. faecalis* per ml brain heart infusion broth					
	148.5 ± 1.5°F		155 ± 1.5°F		158.5 ± 1.5°F	
	Control	100 ppm $NaNO_2$	Control	100 ppm $NaNO_2$	Control	100 ppm $NaNO_2$
0	8.08	8.05	7.08	7.33	6.01	6.26
10	7.68	7.63	6.04	6.45	4.85	4.86
40	6.65	6.68	3.73	2.81	3.75	2.84
70	5.90	6.00	3.73	2.30	3.36	1.23
100	3.83	4.05	3.32	1.69		
130	3.19	3.86	3.20	1.04		

reached, as measured by geometrically centered copper-constantan thermocouples. The remainder of the pack was processed 78 min at 159°F and reached a maximum center temperature of 158°F. The cans were incubated at 50 ± 1°F. Duplicate or triplicate cans were removed periodically and their contents analyzed for the presence of viable enterococci as follows:

Presumptive test. Serial dilutions of samples were placed in tubes of azide dextrose broth (Difco). The tubes were incubated 24 hr at 98°F. Growth indicated presumptive evidence of enterococci.

Confirmed test. A 0.1-ml sample was taken from each positive presumptive tube and inoculated into trypticase soy broth (Difco) containing 6.5% NaCl. The tubes were incubated 24 hr at 113°F, after which the tubes showing growth were examined for gram-positive streptococci. The catalase reaction of these cultures was tested by placing 0.2 ml of culture in a spot plate and adding a few drops of 10% hydrogen peroxide. Absence of bubbles during a 5-min holding period was considered a negative catalase reaction.

RESULTS

The data in Table 1 show that, at 148.5±1.5°F, death rates were similar throughout the test in both nitrite-containing and control cultures. At 155±1.5°F a tenfold difference in viable cell count was apparent less than 40 min after the cultures reached the temperature range. Samples taken at 100 min showed 2,100 survivors per ml in the control culture, and 49 per ml in the nitrite-treated culture. At 130 min, the ratio was 1,600:11. Results were similar at 158.5±1.5°F. Within 70 min of reaching 157°F, the viable count of the nitrite culture had fallen from 1,820,000 to 17 per ml, and that of the control had fallen from 1,030,000 to 2,300.

Table 2 shows the results of experiments with *S. faecium* cultures at 148.5±1.5°F and 158.5±1.5°F. Again, no difference in thermal tolerance was observed at the lower temperatures. At 158.5±1.5°F, however, the nitrite effect was apparent, with 1,300 survivors per ml in the control bottle and only 20 per ml of the culture to which nitrite had been added.

Table 2. Effect of nitrite on survival of stationary phase *Streptococcus faecium* cultures subjected to heat.

Minutes in temperature range before sampling	Log *S. faecium* per ml brain heart infusion broth			
	148 ± 1.5°F		158.5 ± 1.5°F	
	Control	100 ppm $NaNO_2$	Control	100 ppm $NaNO_2$
0	8.00	7.95	6.36	5.98
10	7.51	7.80	5.20	5.40
40	6.76	6.60	3.90	2.97
70	5.59	5.37	3.51	2.51
100	3.66	3.48	3.11	1.30
130	2.57	2.70	2.80	1.08

Ascorbic acid has proved to be extremely useful in enhancing the bright color of cured meats. Since this substance reduces nitrite to nitric oxide under acid conditions (Hollenbeck and Monahan, 1953), it seemed possible that the nitrite effect might be overcome by the simultaneous addition of ascorbic acid to the test system. The data in Table 3 were obtained from an experiment in which *S. faecium* cultures were treated with 100 ppm sodium nitrite, 112 ppm ascorbic acid, and both reagents. The pH of the three test cultures and the control culture were all 6.4±0.05. The temperature chosen was 154.5±1.5°F. The results showed that ascorbic acid, under the conditions of the test, did overcome the ability of nitrite to increase the thermal susceptibility of *S. faecium*.

Table 4 shows the results of a test in which a batch of enterococcus-inoculated canned luncheon meat was divided into two parts and subjected to

Table 3. Reversal of nitrite effect by ascorbic acid.

Minutes @ 154.5 ± 1.5°F	Log *Streptococcus faecium* per ml brain heart infusion broth at 154.5 ± 1.5°F			
	Control	100 ppm $NaNO_2$	112 ppm ascorbic acid	$NaNO_2$ + ascorbic acid
0	7.92	8.19	8.14	8.05
10	7.25	7.44	7.37	7.39
20	4.42	4.08	4.87	4.90
35	3.65	2.00	4.83	4.43
65	2.85	0.00	3.04	3.57

slightly different processes at 159°F. One group of cans was held 78 min at 159°F, attaining a center temperature of 158°F; the other was processed 69 min at 159°F, reaching an internal temperature of 156.5°F. The "78-minute" cans were above 150°F for 35 min; the "69-minute" cans were above 150°F for 28 min. As might be expected from the previous work, the number of surviving enterococci was considerably higher (about sixfold) in the lighter-cooked product than in the companion product. It was extremely interesting to find, however, that, during storage at 50°F, multiplication was substantial within 90 days in the lighter-processed luncheon meat, whereas the enterococci surviving the somewhat longer process died off steadily until they were no longer detectable.

DISCUSSION

The results with the brain heart infusion broth cultures, though reproducible, did not show a logarithmic order of death. White found this to be the case in some of her thermal death work with *S. faecalis* (Richards and White, 1959, White, 1952). Brown *et al.* (1960) obtained a logarithmic death rate at 150°F with cocci isolated from canned ham when the organisms were suspended in broth, but not when the heating menstruum consisted of bromelin-digested meat or comminuted baby-food meat. Young, vigorous cultures are usually used in determining the thermal death characteristics of nonsporing bacteria. The unusually high enterococcus thermal resistance encountered in our experiments can probably be traced to the age of the cultures. Our choice of stationary-phase cultures was based on the grounds that the physiological status of such cultures parallels most nearly that of the enterococci present in cured-meat emulsions at the time of processing. The lactic acid microflora of raw cured-meat emulsions is not typically in exponential growth, and must comprise cells embracing all physiological ages.

Table 4. Fate of enterococci in luncheon meat during storage at 50°F.

Days storage @ 50°F	Enterococci per g of luncheon meat [a]	
	Processed 78 min @ 159°F (max cent T = 158°F)	Processed 69 min @ 159° F (max cent T = 156.5°F)
0	960	5,750
19– 21	500	5,900
48– 50	620	5,300
62– 64	310	2,000
89– 91	115	12,150,000
94– 96	20	8,000,000
111–113	10	
124–126	1	
158–160	<0.3	

[a] Average of 2–3 cans per sampling period.

Perishable canned cured pork products are processed at heating schedules that result in maximum internal temperatures in the 150–160°F range. This ensures destruction of Trichinae and is optimal for yield and general organoleptic quality.

The data suggest that the specifics of the process affect profoundly the concentration of enterococci in the finished product and the ultimate fate of these organisms during refrigerated storage. The longer such products are in excess of 150°F during thermal processing, the greater will be the synergistic enterococci-killing effect of the nitrite in the emulsion.

The difference in the fate of enterococci surviving the thermal treatments applied in the inoculated cured-meat pack deserves further consideration. The viable enterococcus concentration of product processed to an internal temperature of 158°F declined steadily during storage at 50°F. Eventually (after about 5 months of incubation), no enterococci were recoverable from the product. Cans prepared from the same emulsion, held a shorter time in the same processing tank and reaching a center temperature of 156.5°, developed high numbers of enterococci—and acid spoilage—within 90 days. It is thus apparent that nitrite, in combination with a crititcal amount of ther-

mal energy, can render enterococci incapable of outgrowth in perishable cured meats—a situation not unlike the thermal injury effect described by Silliker *et al.* (1958) in regard to bacterial spores in shelf-stable cured-meat products. Much of the stability inherent in foods receiving less than a sterilizing thermal process may be traceable to sublethal reactions that prevent outgrowth in the microenvironment of the survivors. Ability to grow in subculture is by no means a definite indication that an organism can grow in the product unit from which it was taken.

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Growth and Toxin Production by Type E Clostridium Botulinum Below 40°F [a]

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(Manuscript received May 7, 1961)

SUMMARY

Mildly heat-shocked spores of four strains of Type E *Clostridium botulinum* (VH, Beluga, Iwanai, and 8E) were inoculated into a heat-sterilized beef stew substrate and incubated at 34, 36, and 38°F. Inoculum levels of approx 4–12 million spores per tube were used with different strains. At 38°F, toxin production and visible outgrowth as evidenced by gas formation beneath a vaspar seal occurred in one or more tubes of each of the strains within 31–45 days. No evidence of growth was visible after 14 days at 38°F, and at this time duplicate tubes of each strain were not toxic. After 31 days, strains Iwanai and Beluga showed 2 of 8 tubes with gas and toxin. None of 8 tubes of the VH and 8E strains showed gas after 31 days. Two tubes of each strain were not toxic. After 45 days, tubes of the VH and 8E strains showed visible gas production and toxin. Inoculated tubes incubated at 34 or 36°F showed no gas production during 104 days. Samples of all strains at both temperatures were toxin assayed at 54 days and 104 days, and toxin was absent in all cases. These results suggest a rather sharply defined critical temperature above and below which spores of Type E *Cl. botulinum* are or are not capable of germinating and initiating toxic outgrowth. The significance of these results in relation to extended refrigerated storage of food products is discussed.

INTRODUCTION

Dolman *et al.* (1950) reported growth and toxin production of the VH strain of Type E *Cl. botulinum* at 43°F. Ohye and Scott (1957) presented a detailed study of the effect of temperature on the growth rates of 10 strains of Type E *Cl. botulinum*. Also, using a cooked meat medium they found growth initiation from spore inocula in 3–4 weeks at 41°F, with toxin detectable after 8 weeks of incubation. Six other strains showed growth, but the cultures did not kill mice although some appeared to produce transient symptoms. However, since this work was done before recognition of the potentiation of Type E toxin by trypsin (Duff *et al.* 1956), the complete absence of toxin in any of these cultures is uncertain. Schmidt *et al.* (1961a) reported that small numbers of Type E spores permitted to survive radiation pasteurization doses were able to germinate and produce toxic outgrowth at 43 and 49°F during comparatively short periods of storage. Strains VH, Beluga, and Iwanai were used in that work, and it was also shown that mildly heat-shocked spore inocula germinated and produced toxic outgrowth at those temperatures. Schmidt *et al.* (1961a) discussed the results of experiments from 3 different laboratories in relation to the extension of the refrigerated storage life of food products by radiation pasteurization. Ohye and Scott (1957) found no growth initiation of 10 Type E strains incubated 22 weeks at 36°F. However, it must be pointed out that those experiments consisted of duplicate tubes each inoculated with 10,000 spores of the strain being studied. Schmidt *et al.* (1961a) also suggested that the implications of the various results obtained to date make it desirable that an intensive study be conducted to determine the absolute minimum temperature that permits outgrowth of Type E spores using high-spore-level inocula. This paper pre-

[a] Presented at the 21st Annual Meeting of the Institute of Food Technologists, May 8, 1961.

sents the results of studies that attempt in a preliminary way to determine the ability for outgrowth at 34, 36, and 38°F of heat-shocked spores of 4 strains of *Cl. botulinum* Type E.

EXPERIMENTAL METHODS

Spore suspensions. The spore suspensions used as inocula were dilutions of stock suspensions prepared and used for determining radiation resistance as described by Schmidt *et al.* (1961b). Portions of the stock suspensions were centrifuged and washed three times by resuspending in sterile water and centrifuging at 6000 rpm in a Servall angle centrifuge. After the third washing the sedimented spores were resuspended to the original volume, heat-shocked for 13 min at 140°F, and diluted 10-fold to provide the final inoculum.

Substrate. Frozen beef stew used as a substrate for determination of the radiation resistance of spores of Type E *Cl. botulinum* (Schmidt *et al.*, 1961b) was thawed and distributed in approximately 30-g quantities in 25 × 150-mm screw-cap tubes. After the addition of 5 ml of water, the tubes were sterilized for 15 min at 250°F.

Inoculation. Tubes of the sterilized beef stew were inoculated with 1-ml quantities of suspension and sealed with a layer of sterile vaspar. The tubes were then immersed 5 min in water at 140°F to reset the vaspar into a smooth layer, and then chilled for about 1 hr in cold tap water. Following this, the tubes were held at least 2 hr in ice water with melting ice. The tubes were dried rapidly and placed at the desired incubation temperature in Lo-Temp BOD incubators. For each incubation temperature, 10 tubes of the beef stew substrate were inoculated with each strain tested. The inoculum level per tube varied between 4 million and 12 million for the different strains.

Incubation and observation. Sets of tubes for each of the 4 strains tested were incubated at 34, 36, and 38°F. The tubes incubated at 38°F were examined each week, and those incubated at 34 and 36°F were examined every two weeks. The tubes were removed from the incubator and examined for visible gas production beneath the vaspar seal. This observation could be conducted quite readily by tapping the sides of a pair of tubes against each other. Since complete observation of a rack of 40 tubes required less than 5 min, there was little opportunity for a significant temperature rise in a 25-mm tube of this quite viscous product.

Sampling and toxin assay. After certain periods of incubation, samples were removed from the incubators and assayed for Type E toxin. The vaspar seal was removed and the contents of the tubes blended 1:1 with sterile water. Toxin assay was conducted using trypsin digestion as described by Schmidt *et al.* (1961b). Tubes showing no gas at the respective incubation temperatures at the time of sampling were placed in water at 95°F for at least 2 hr to determine whether gas was detectable under these conditions.

RESULTS AND DISCUSSION

Tables 1 and 2 show the results with the spore inocula of 4 different strains incubated at 38°F. After 14 days' incubation no gas was detectable either in 10 tubes of each strain at 38°F or in 2 tubes of each strain subsequently warmed to 95°F. These 2 tubes of each strain also were nontoxic after this period of incubation. After 31 days of incubation, 2 tubes of the Iwanai strain and one tube of the Beluga strain showed definite evidence of gas production at 38°F. The tube of the Beluga strain and one of the Iwanai tubes showed detectable toxin. After 45 days of incubation additional tubes of the Iwanai and Beluga strains showed gas at 38°F, as did also 2 tubes of the VH strain and one tube of the 8E strain. All tubes showing gas at 38°F after 45 days' incubation were shown to contain Type E toxin. After 31 and 45 days of incubation, when tubes of the strains showing gas were toxin assayed, a tube not showing gas at 38°F was also tested by warming to 95°F and then assayed for toxin. Tubes of the Type 8E strain that showed no gas at 38°F after 60 days of incubation were also tested by this procedure. The results of these tests are included in Tables 1 and 2 and summarized in Table 3. The data in Tables 1 and 2 show that, with each strain tested, toxic outgrowth with visible gas production can occur within 31–45 days at 38°F in at least a part of 10 replicate samples inoculated. Other replicate tubes of the same inoculum may require 53–60 days at 38°F to produce detectable gas. The data summarized in Table 3 show that 2 of the 4 strains after 45 days of incubation may produce an amount of gas not detectable at 38°F but detectable upon warming to 95°F. The 8E strain requires about 60 days of incubation to produce the same result. Also, with 3 of the strains at certain periods of incubation, it is evident that a detectable toxin titer may

Table 1. Gas formation and toxin production from spore inocula of strains VH and Iwanai incubated at 38°F.

Strain	Tube no.	Days incubation		Gas at 95°F	Toxin assay	
		For gas production	Before toxin assay		Results [b]	Dilution level tested
VH	1	0/14	14	0	NT	80
	2	0/14	14	0	NT	80
	3	0/31	31	0	T	80
	4	0/31	31	0	NT	8
	5	53	—[a]	—	—	—
	6	45	45	—	T	80
	7	45	—	—	—	—
	8	0/45	45	+	T	80
	9	53	—	—	—	—
	10	60	60	—	T	80
Iwanai	1	0/14	14	0	NT	80
	2	0/14	14	0	NT	80
	3	31	31	—	T	80
	4	31	31	—	NT	8
	5	38	45	—	T	80
	6	0/45	45	+	T	8
	7	45	—	—	—	—
	8	45	—	—	—	—
	9	47	—	—	—	—
	10	47	—	—	—	—

[a] Not tested.
[b] NT = Non-toxic, T = Toxic.

Table 2. Gas formation and toxin production from spore inocula of strains Beluga and 8E incubated at 38°F.

Strain	Tube no.	Days incubation		Gas at 95°F	Toxin assay	
		For gas production	Before toxin assay		Results [b]	Dilution level tested
Beluga	1	0/14	14	0	NT	80
	2	0/14	14	0	NT	80
	3	31	31	—	T	80
	4	0/31	31	0	NT	8
	5	38	45	—	T	80
	6	0/45	45	0	T	8
	7	38	—[a]	—	—	—
	8	47	—	—	—	—
	9	53	—	—	—	—
	10	53	—	—	—	—
8E	1	0/14	14	0	NT	80
	2	0/14	14	0	NT	80
	3	0/31	31	0	NT	8
	4	0/31	31	0	NT	8
	5	45	45	—	T	80
	6	0/45	45	0	T	8
	7	53	—	—	—	—
	8	53	60	—	T	80
	9	0/60	60	0	T	8
	10	0/60	60	+	T	80

[a] Not tested.
[b] NT = non-toxic, T = toxic.

Table 3. Summary of toxin assay of samples that showed no gas at 38°F at time of examination.

Strain	Tube no.	Incubation time (days)	Gas at 95°F	Toxin assay	
				Result	Dilution level tested
VH	3	31	0	T	80
	8	45	+	T	80
Iwanai	9	45	+	T	8
Beluga	7	45	0	T	8
8E	6	45	0	T	8
	9	60	0	T	8
	10	60	+	T	80

exist without gas being apparent even at 95°F.

At 36°F, 3 tubes of each strain were tested following 42 days of incubation, 2 tubes following 54 days, and 5 tubes following 104 days. At 34°F, 3 tubes were examined after 54 days, and 7 tubes after 104 days. None of the tubes showed gas either at the respective incubation temperatures or after warming to 95°F. All tubes of strains Iwanai, Beluga, and 8E were negative for toxin at a level equivalent to 8 MLD per gram. Some of the tubes of the VH strain showed toxicity at a level equivalent to 8MLD, but all samples were negative at 16 MLD. It was subsequently shown that, in spite of washing and preheating, there was a slight carryover of trypsin-digestible toxin in the inoculum. The washed preheated suspension of the VH strain contained approx. 200 MLD per ml following trypsin digestion. When one ml was inoculated into tubes containing 35 g of substrate, this would account for a toxin level of approx. 8 MLD per gram, as was found with this strain. The results obtained at 36°F provided some confirmation of the data of Ohye and Scott (1957), who found no growth of 10 strains in 22 weeks at 36°F, using much lower spore inocula levels.

These experiments seem to indicate conclusively that a refrigerated storage temperature of 38°F is not safe over extended periods with respect to preventing outgrowth and toxin production by spore inocula of Type E *Cl. botulinum*. It could be expected that somewhat less optimal culture media and lower spore inocula levels might somewhat mitigate the hazard of outgrowth at this temperature, or markedly delay the time at which it occurs. The effect of such factors remains to be revealed by future investigation. At present one must hesitate to suggest the storage of food products capable of supporting growth of Type E *Cl. botulinum* at a maximum temperature of 38°F beyond a storage time of approx 3 weeks. The tests conducted at 34 and 36°F, within the limits of the small number of strains and the spore inoculum level of each used, indicate a significant and very favorable effect of these temperatures in preventing the hazard of Type E botulism in refrigerated foodstuffs. Comparison of the results at 38 and 36°F, however, suggests a very sharply defined critical temperature zone above which spores of Type E *Cl. botulinum* can present a public health hazard during moderately long storage periods. The very narrow borders of the upper and lower limits of this zone must make us wary of specifying too definitely on limited evidence the temperature at which the hazard of Type E botulism no longer occurs until much more supporting evidence is at hand. The data presented indicate in a preliminary fashion some of the problems of the Type E botulism hazard that may be involved in extended refrigerated storage life of foodstuffs. It is our hope that these results will stimulate considerable additional investigation in this field in the near future.

Schmidt *et al.* (1961a), in an experiment preliminary to determining the ability of spores surviving irradiation to germinate and result in outgrowth at refrigeration temperatures, tested the ability of mildly heat-shocked spores to germinate and develop in beef stew at 43 and 49°F. Approximately the same inoculum level of spores and procedure were used as in this work with strains VH, Iwanai, and Beluga. Gas production was detectable in 10–12 days at 49°F, and in 19–22 days at 43°F. The average time for gas production was estimated as 11 days at 49°F and 20 days at 43°F. In the work now presented, we may estimate 36 days as the average time at which some tubes of each of the 4 suspensions produced gas at 38°F. Fig. 1 shows the relationship that develops when the average time for detectable gas is plotted on a logarithmic scale against degrees Fahrenheit on a linear scale. It is perhaps surprising that such a complex phenomenon

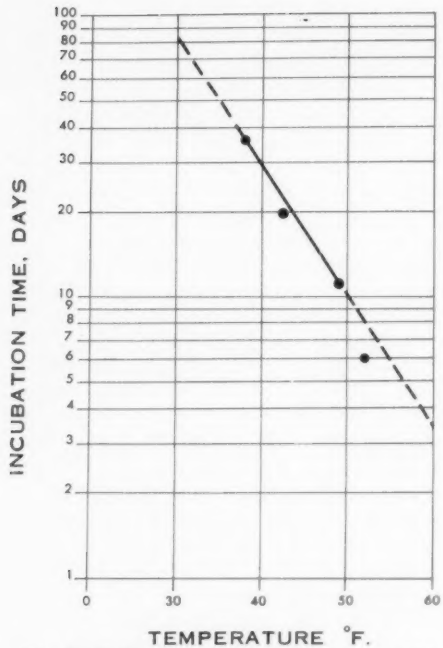

Fig. 1. Relation of incubation time for visible gas production and incubation temperature for 4 strains of *Clostridium botulinum* Type E.

as the appearance of detectable gas, which is dependent on spore germination, cell growth, and gas formation, as well as gas solubility and gas pressure at each temperature, should appear so regularly related to temperatures between 38 and 49°F. An attempt was made to establish another point on the curve by conducting a test with the same inoculum with 5 tubes of each strain incubated at 52°F. In this case gas was detectable in tubes of all strains between 5 and 7 days, so that the estimated average time of 6 days falls somewhat below the extrapolated curve. The extrapolated portion below 38°F has been shown to be invalid by the results of our work at 34 and 36°F. However, the extrapolation did have some value in suggesting incubation time intervals at which tubes that exhibited no gas at the respective incubation temperatures should be warmed to 95°F so that small quantities of gas might be detected and also assayed for toxin. If this relationship between time for detectable gas production and temperature holds for spores of additional strains in various substrates, it should be possible to conduct surveys for their behavior quite rapidly at 2 temperatures between 40 and 50°F. The line extrapolated below 40°F could then be used as a guide for further observations requiring prolonged incubation.

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Some Characteristics of Coagulase-Positive Staphylococci from Market Meats Relative to Their Origins Into the Meats [a, b]

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(Manuscript received July 1, 1961)

SUMMARY

From four market meats (hamburger, pork chops, beef liver, and round steak) obtained at 28 retail stores, 136 isolates of coagulase-positive staphylococci were recovered. Suggestive of human sources of *Staphylococcus aureus* to the meats were bacteriophage types of most of the isolates similar to those of human strains. Also, over 70% of the isolates produced hemolysins characteristic of human strains of *S. aureus*. Suggestive of non-human sources was the finding that none of the strains was resistant to penicillin and three other antibiotics and that 6% were resistant to streptomycin, 3% to chlortetracycline, and 12.5% to ristocetin.

The distribution of potentially virulent *Staphylococcus aureus* strains has recently been shown by many investigators to be widespread. In brief, it has been shown that these organisms exist on almost every type of object and in most all environments where man and certain animals exist (Walter *et al.*, 1958; Fratta and Mann, 1960; Clark, Moore, and Nelson, 1961). Such widespread occurrence might be either consequences of or contributing factors in the spread of these strains in human outbreaks of staphylococcal infections, which are so widespread. Foods, unfrozen meats in particular, seem to have been overlooked as possible sources of spread of these organisms. A first consideration in this regard should perhaps be a survey of market meats to determine their content of coagulase-positive *S. aureus* and whether the numbers are sufficient to be of possible significance in their spread. One such story has been reported (Jay, 1961). A second consideration should be a characterization of the market staphylococci to determine whether they are similar to strains in human outbreaks and whether they are capable of initiating infection in man.

This paper reports the bacteriophage and hemolysin patterns and the antibiotic sensitivity of coagulase-positive *S. aureus* from 4 market meats.

MATERIALS AND METHODS

The fresh meats chosen for study were: hamburger or ground beef, round beef steak, beef or calf liver, and pork chops. The first 3 meats were chosen primarily because they are usually cooked to various degrees of doneness and therefore present perhaps the greatest chance for the spread of staphylococci to the purchaser, the cook, and the consumer.

The meats were purchased over the counter from 28 retail grocery stores and meat markets selected at random in the Baton Rouge area. The meats were bought in ordinary lots without informing the clerk of intended use. On the day of purchase, they were treated as follows: 50-g samples were taken primarily from the surface, homogenized in Waring blenders with saline, and 0.1-ml portions were planted on the surface of staphylococcus medium #110 (Difco) fortified with egg yolk, tellurite-glycine agar, and mannitol

[a] This study was supported by research grant E-3089 from the Institute of Allergy and Infectious Disease, National Institutes of Health, U. S. Public Health Service.

[b] Presented in part at the 61st annual meeting of the American Society for Microbiology, April, 1961.

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salt agar. Colonies resembling coagulase-positive *S. aureus* were selected from all three media, coagulase tested with Bacto-coagulase plasma, and phage typed by the method of Blair and Carr (1960). In addition to the basic set of 21 phages, phages 39, 42B, 44A, 70, 81, 83, and 523 were used, making a total of 28. The basic set of propagating strains, along with numbers 81 and 83, were supplied by Dr. John E. Blair. The other 5 strains were supplied by Dr. Jack N. Baldwin.

The production of the alpha, beta, and delta hemolysins was determined with the agar-plate technique described by Bryce and Rountree (1936), Williams and Harper (1947), and Elek and Levy (1950). Alpha hemolysin clears rabbit and sheep red-blood cells; beta lysin attacks sheep cells, is "hot-cold," and does not lyse rabbit cells; and delta lysin attacks rabbit, sheep, and human blood cells. Antibiotic sensitivity tests were run by the 3-disc technique, per Difco instructions, with the following 8 antibiotics: penicillin, streptomycin, chlortetracycline (CTC), chloramphenicol, novobiocin, erythromycin, kanamycin, and ristocetin. Resistant and moderately sensitive cultures were further tested by the tube dilution method.

RESULTS AND DISCUSSION

From the 3 media employed, 136 isolates of coagulase-positive staphylococci were recovered. Of these, 97 (71%) were phage-typable, representing 32 phage strains. The phage groups are given in Table 1. The largest number of typables belonged to Group III (55%), followed by the mixed group. Five reports taken at random from the literature on hospital staphylococci showed an average of 53% for Group III (range 37–68%). Of interest here is the fact that a large number of food-poisoning strains have been shown to fall into Group III. Twenty-seven percent of the typable isolates fell into the mixed group and consisted for the most part of Groups I and III. Ten percent belonged to Group I, and 5 and 3% to Groups II and IV, respectively. Papavassiliou and Obiger (1959) found that 68% of 84 phage-typable staphylococci recovered from milk belonged to Group III, and 29% belonged to a mixed group composed of III and IV types.

Table 1. Distribution of the typable staphylococci recovered from market meats within the bacteriophage groups.

Group	Number	Percent
I	10	10
II	5	5
III	53	55
IV	3	3
Mixed [a]	26	27

[a] Mostly Groups I and III.

Table 2. Numbers of isolates of coagulase-positive staphylococci and phage strains from the 28 meat markets.

Market No.	No. of isolates		No. of phage strains
	Typable	Nontypable	
1	5	5	4
3	2	0	1
6	5	1	4
7	15	12	8
8	6	2	4
9	1	1	1
11	3	3	2
12	9	2	1
13	2	3	1
14	7	0	2
16	5	0	4
17	13	1	2
18	1	0	1
19	4	0	2
20	6	3	4
21	3	1	2
22	1	0	1
23	2	0	1
24	1	0	1
25	1	1	1
28	5	4	4
Totals	97	39	

Of the 28 meat markets, 21 (75%) yielded meats from which coagulase-positive *S. aureus* was recovered. Table 2 shows the number of isolates from each market and the number of phage strains represented. The largest number was recovered from market no. 7: 15 typable and 12 nontypable. This number was composed of 8 phage strains. This market is an all-meat market, specializing in low-priced bulk-rate meats. A large number of phage strains from one market is taken to be indicative of several sources of the organisms in the meats. Market no. 17 yielded 13 typable isolates representing only 2 strains. This, on the other hand, probably indicates that these

organisms originated from a fewer number of sources, perhaps only one. In general, however, the larger the number of isolates the greater was the number of different phage strains. Of the 7 stores from which staphylococci could not be recovered, 3 were meat markets, 2 were chain stores, and 2 were neighborhood grocery stores.

The most frequently encountered phage patterns are shown in Table 3. Of the types

Table 3. Phage patterns of *S. aureus* from market meats.

Pattern	Total No. isolates	No. markets recovered from
52A, 52A/80, 44A/52A/80	10	7
54	9	7
83(Va4)	7	2
6/47/53/54/75/83	7	1
7	4	2
77	3	3
53/83	3	3
42D	2	1
42E	2	2
81	2	2
22 others	48	1 each

52A, 52A/80, and 44A/52A/80, which all seem closely related, the total recovered was 10 from 7 different markets and about equally distributed among the 4 meats. Type 54 was the next-most frequently encountered pattern, followed by 83 (Va4), 6/47/53/54/75/83, 7, 77, 53/83, 42D, 42E, and 81. One isolate each of 22 other patterns was recovered, making a total of 32 phage strains. Of the phage patterns shown in Table 3, 35% were recovered from hamburger and 30% from pork chops, while only 18% came from steak and liver. At least 8 of the 12 patterns shown have at one time or another been involved in human outbreaks. At least 21 of these 32 patterns have been recovered from humans. No isolate of the pattern 80/81 was recovered.

The numbers of *S. aureus* recovered from the 4 meats are shown in Table 4. Hamburger yielded the largest number, followed by steak, pork chops, and beef liver. Although the liver yielded the fewest isolates, it yielded the most phage strains.

Table 4. Meat source of staphylococcal isolates from 28 markets.

Meat	Total isolates		No. of phage strains
	Typable	Nontypable	
Hamburger	30	11	15
Pork chops	26	6	18
Beef liver	23	6	21
Steak	18	16	13

The hemolysin patterns of all 136 isolates and of the predominant phage types are shown in Table 5. Seventy-six produced the alpha-delta lysins, 28 the alpha-beta-delta, 22 the alpha, and 2 the alpha-beta lysins, and 8 were nil in these regards. Of the most predominant phage patterns, 58% were alpha-delta producers, 26% alpha-beta-delta, 14% alpha, and 2 were nil. Elek and Levy (1950) reported that approximately 75% of animal strains produced the beta lysin whereas only 10% produced the alpha-delta lysins. Only 11% of human strains were reported to produce the beta hemolysin. Clark *et al.* (1961) found that 49 of 51 strains of *S. aureus* recovered from bulk-tank raw-milk samples produced either alpha-beta or beta lysins. Richou *et al.* (1959) concluded, primarily from hemolysin and fibrinolysin production, that the coagulase-positive staphylococci they recovered from food products were not of human origin.

The sensitivity of these organisms to 8

Table 5. Hemolysin pattern of all isolates and of predominant phage patterns.

Phage pattern	Total	Hemolysins				
		α	$\alpha\delta$	$\alpha\beta$	$\alpha\beta\delta$	Nil
All isolates	136	22	76	2	28	8
52A, 52A/80, 44A/52A/80	10	3	3	.....	3	1
54	9	.....	6	.....	3	.....
6/47/53/54/75/83	7	.....	7	.....	.....	.....
83(Va4)	8	2	5	.....	1	.....
7	4	1	1	.....	2	.....
53/83	3	.....	2	.....	1	.....
42D	2	.....	1	.....	1	.....
Totals*		6	25		11	1
Percent of types indicated		14	58		26	2

* Totals of the phage types indicated only.

antibiotics is recorded in Table 6. All isolates were sensitive to penicillin, erythromycin, chloramphenicol, and kanamycin. Three of the 136 were resistant to novobiocin, 4 to CTC, 8 to streptomycin, and 17 (12.5%) to ristocetin. The phage patterns of the resistant strains indicate that 40% of them were nontypable whereas approx 40% belonged to Group III, the largest number being type 83. The relatively few antibiotic-resistant isolates, especially to streptomycin and CTC, with none being resistant to penicillin, also suggests that many of these organisms probably had origins into the meats other than human sources.

CONCLUSIONS

It is somewhat difficult to draw conclusions from these data as to what is the main source of *Staphylococcus aureus* into market meats and the actual role of market meats in the possible spread of staphylococci in the population. On the one hand, most of the phage patterns recovered have been at times involved in human outbreaks, and most of them produced hemolysins characteristic of human strains of *S. aureus*. These two findings indicate human sources. On the other hand, the antibiotic resistance was considerably lower than is usually found in clinical isolates. Also, the rather wide distribution of the strains among the markets, the occurrence of 21% with animal hemolysin patterns, and the relatively small numbers recovered all tend to suggest that their sources were other than human. It is quite likely that both sources are involved, with the human source perhaps contributing the smaller number.

Table 6. Antibiotic sensitivity of the staphylococci and phage patterns of the resistant strains.

Antibiotics	No. sensitive	% sensitive	Phage pattern of resistant
Penicillin	136	100	.....
Erythromycin	136	100	.....
Chloramphenicol	136	100	.....
Kanamycin	136	100	.....
Novobiocin	133	97.8	7, (2)* Nontypable
CTC	132	97.1	81, (3) Nontypable
Streptomycin	128	94.1	(2) 83, 52A, 77, 44A/52A/80, 3C/6/29/44A/53/80 (2) Nontypable
Ristocetin	119	87.5	(4) 83, (2) 53/83, 3C, 52A/80, 54, 54/55, 53/54/80, (6) Nontypable

* Number of strains.

ACKNOWLEDGMENTS

The technical assistance of Joe N. Hobbs, Betty S. Stephens, and Ella E. Turner is gratefully acknowledged.

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Media for Detecting Pectolytic Gram-Negative Bacteria Associated with the Softening of Cucumbers, Olives, and Other Plant Tissues[a]

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(Manuscript received May 29, 1961)

SUMMARY

The limitations of eosine methylene blue or basic fuchsin polypectate gels prompted further investigations to develop a selective pectate gel that could be used to enumerate or isolate gram-negative pectolytic bacteria even when they composed a minor portion of the microbial population.

Crystal violet and brilliant green with bile proved the most effective of several selective agents studied. Of the two, crystal violet was much less inhibitory. Actidione, 10 mg per L added after sterilization, is helpful in the control of growth of molds and yeasts on this medium.

The crystal violet medium has a productivity comparable to that of standard plate-count agar when pure cultures are used. It was successfully used to enumerate known populations of pectolytic gram-negative bacteria among mixed populations of other nonpectolytic gram-negative and -positive bacteria. It is also being used routinely for enumeration and isolation of pectolytic gram-negative bacteria from natural sources.

Thus far, pectolytic bacteria belonging to 5 genera, including *Aerobacter*, *Escherichia*, *Paracolobactrum*, *Aeromonas*, and *Achromobacter*, have been isolated by use of the crystal violet gel.

Vaughn *et al.* (1957) described the first attempts to devise selective pectate gel media for detection of gram-negative pectolytic microorganisms associated with softening of cucumbers, olives, and other plant tissues. In investigations of the cause of a characteristic softening of olives known as "sloughing" it became apparent to the present authors that the selective eosine, methylene blue, and basic fuchsin gels used by Vaughn *et al.* (1957) were sometimes unsatisfactory. If the gram-negative bacteria predominated the populations, no trouble was encountered with the selectivity of these gels. When species of *Bacillus* were the most abundant, however, isolating gram-negative pectolytic bacteria became impossible.

Pectinous gel media described by Funk (1937), Baier and Manchester (1943), McCready *et al.* (1943), Jones (1946), and Wieringa (1949), also permitted indiscriminate growth of microorganisms. Therefore, the present study was undertaken to develop a medium that would be selective for detection and enumeration of gram-negative pectolytic bacteria under conditions where they might constitute a minor portion of the natural pectolytic microbial population.

EXPERIMENTAL METHODS

Test culture collection. A total of 132 pure cultures of different microbes were used as test organisms. These included 76 identified bacteria, 21 identified molds, and 16 identified yeasts, respectively representing 18, 13, and 7 genera. The microorganisms are listed in Tables 1 and 2. Used as controls were 19 partially identified cultures of pectolytic gram-negative bacteria: 3 *Achromobacter*, 9 *Aerobacter*, 5 *Aeromonas*, 1 *Escherichia*, and 1 *Paracolobactrum*.

[a] Presented at the 21st annual meeting of the Institute of Food Technologists, New York, May, 1961.

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Constituents used in media. Bacto-peptone (Difco) was used as the source of organic nitrogen and other accessory nutrients. Potassium phosphates (K_2HPO_4 and KH_2PO_4) were used to buffer the medium at pH 7.0 because bacterial "polygalacturonases" are most active at pH values between 7.5 and 9.4 (Fernando, 1937; Wood, 1951, 1955; Nortje and Vaughn, 1953; Smith, 1958; and Nagel and Vaughn, 1961a).

Calcium chloride was used in all test media. Calcium ion is required to activate some pectolytic enzymes (Wood, 1951, 1955; Smith, 1958; Nagel and Vaughn, 1961a). The calcium acts also to form a stronger gel through the addition of chemical salt bonds that help strengthen the hydrogen bonds.

Various inhibitory agents were added to suppress the growth of all but the gram-negative bacteria (see below). Crystalline actidione (Upjohn Co.), an antibiotic produced by *Streptomyces griseus*, was used in some of the test media to inhibit yeasts and molds. This antibiotic is active against many fungi, but has no effect on bacteria in the concentrations tested by Whiffen (1948), Phillips and Hanel (1950), Jeffers (1954), and Green (1955). Phillips and Hanel (1950), Green (1955), and Klis *et al.* (1957) have suggested that actidione be used in media to inhibit the growth of yeasts and molds to facilitate the isolation of bacteria.

An aqueous actidione solution (10 mg/ml) was sterilized with a Millipore filter and added to the sterilized and cooled (50–55°C) media and thoroughly mixed. The antibiotic was used in a final concentration of 10 mg/L of medium.

The sodium polypectate used in these studies was a purified alkaline degradation product of citrus pectin produced by Sunkist Growers, Ontario, California. This product was completely demethylated and contained less than 1.0% total invert sugars (Beisel, 1960).

Preparation of gels. The sodium polypectate was weighed separately. Other constituents were added to the desired amount of boiling distilled water. It was advantageous to add the inhibitory dyes, calcium chloride, actidione, sodium lauryl sulfate, and other compounds required in small quantities from concentrated alcoholic or aqueous solutions to reduce errors in weighing and to conserve time.

The hot solution containing all of the desired ingredients was placed in a Waring Blendor in 500-ml portions, and the required amount of the polypectate was added in small increments while the solution was slowly stirred. A rheostat was used to control the motor speed of the Blendor to reduce the amount of occluded air.

After mixing (3 min or more), the 500 ml of homogeneous slurry was transferred to a 1-L Erlenmeyer flask. The medium was then deaerated by placing it in a steam cabinet for approx 15 min or subjecting it to a vacuum rapidly released several times in order to remove entrapped air. Deaeration is essential to prevent undue frothing during subsequent sterilization (15 min at 120°C). Prolonged heating tends to break down the polymer, thus weakening its gel strength.

Upon cooling, the medium was poured into standard Petri plates. If actidione was used, a measured amount of the sterilized antibiotic was added to the cooled (50–55°C) medium and thoroughly mixed before pouring. Since the liquid medium was quite viscous, it was necessary to pour the plates at 45°C or above.

The gel can be streaked 4 hr after being poured, but it is firmer if left overnight at room temperature. Plates of gel media should be used while fresh because the medium dries on storage and organisms do not readily show pectolysis. If test tubes were used, the medium was poured into the tubes and then deaerated and sterilized as described above.

EXPERIMENTAL RESULTS

The development of selective media. In all of the studies on the effect of selective inhibitory substances, the following standard basal medium was used: 5.0 g peptone (Difco), 5.0 g K_2HPO_4, 1.0 g KH_2PO_4 0.6 $CaCl_2 \cdot {}_2H_2O$, 70.0 g sodium polypectate (Exchange Product No. 24), 1000 ml distilled water.

Plates of test medium were streaked with unclassified cultures including 19 gram-negative and 5 gram-positive rod-shaped bacteria, 3 gram-positive cocci, and 2 yeast strains. A number of known inhibitory substances were tested alone and in various combinations, in this medium at different pH values.

In the preliminary experiments, brom thymol blue was used at a concentration of 0.025 g/L at pH 6.4 and 7.0. A combination of crystal violet, eosine-y, and methylene blue was tried in respective concentrations of 0.004, 0.4, and 0.065 g/L. Crystal violet was tested alone at 0.004 g/L, as was brilliant green at 0.0132 g/L. Brilliant green was also tested in the presence of 20 g Bacto-oxgall/L. Crystal violet (0.004 g/L) was also tested in the presence of 0.1 g/L of sodium lauryl sulfate. In addition, sodium lauryl sulfate was used alone at 0.2 g/L. Basic fuchsin was tested in concentrations of 0.015 g/L and 0.0075 g/L. Eosine-y and methylene blue were tested in respective combinations of 0.4 and 0.065 g/L.

The results of these preliminary experiments indicated none of these media were selective enough

Table 1. Effect of selective polypectate media on growth of mold and yeast.[a]

	Brilliant green bile	Crystal violet	Brilliant green bile	Crystal violet
			Plus 10 mg/L actidione	
Molds				
Alternaria solani	—	—	—	—
Aspergillus glaucus	—	x	—	x
Aspergillus niger	xxD	x	x	x
Aspergillus sp. (yellow)			—	—
Aspergillus sp. (black)			—	—
Byssochlamys fulva	—	—	—	—
Geotrichum candidum	xx	x	x	x
Geotrichum candidum	xD	xD	x	x
Geotrichum candidum	xxD	xxD	x	x
Geotrichum candidum	xxD	xxD	x	—
Nematospora coryli	—	—	—	—
Oospora lactis	xD	xxD	x	x
Penicillium chrysogenum	—	—	xx	x
Penicillium sp.	xD	xD	xx	—
Pericystis apis			—	—
Prototheca sp.	—	—	—	—
Pullularia pullulans	x	—	—	—
Pullularia pullulans	—	x	—	x
Taphrina deformans	—	—	—	—
Trichosporon cutaneum	xx	xx	xx	x
Ustilago sp.	—	x	—	—
Yeasts				
Candida guilliermondii	x	—	—	—
Candida krusei	x	x	—	—
Candida parapsilosis	xxx	—	—	—
Debaryomyces guilliermondii	—	—	—	—
Hanseniaspora valbyensis	—	—	—	—
Rhodotorula gultinis	—	—	—	—
Rhodotorula mucilaginosa	x	x	x	x
Saccharomyces acidifaciens	x	—	—	—
Saccharomyces cerevisiae	—	—	—	—
Saccharomyces cerevisiae	—	—	—	—
Saccharomyces cerevisiae	x	—	—	—
Saccharomyces fragilis			—	—
Saccharomyces rouxii	x	—	—	—
Sporobolomyces salmonicolor	—	—	—	—
Sporobolomyces salmonicolor	x	—	—	—
Sporobolomyces sp.	—	—	—	—
Torulopsis magnoliae	xx	—	—	—

[a] — no growth; x growth limited to area at beginning of streak; xx moderate growth; xxx profuse growth; D depressions.

to eliminate the growth of all the gram-positive bacteria. Both brilliant green and crystal violet were promising, however, and it was decided to concentrate on these two dyes.

Further studies with brilliant green and crystal violet. Crystal violet was used in concentrations of 0.0016–0.04 g/L. Brilliant green was tested at concentrations varying from 0.0264 to 0.0369 g/L. The brilliant green media were supplemented with Bacto-oxgall. It was found that concentrations of 0.02 g/L of crystal violet or 0.0264 g/L of brilliant green plus 20 g/L of Bacto-oxgall resulted in selectivity for the differential growth of the pectolytic gram-negative test bacteria. The ingredients of these two media were:

Brilliant green bile polypectate gel: 5.0 g peptone (Difco), 8.0 g K_2HPO_4, 20.0 g Oxgall (Difco), 0.6 g $CaCl_2 \cdot 2H_2O$, 0.0264 g brilliant green, 70 g sodium polypectate (Exchange), and 1000 ml distilled water.

Crystal violet polypectate gel: 5.0 g peptone (Difco), 5.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.6 g $CaCl_2 \cdot 2H_2O$, 0.02 g crystal violet, 70.0 g sodium polypectate (Exchange), and 1000 ml distilled water.

Pectolysis resulted in a softening of the media. Crateriform depressions were always formed surrounding discrete colonies of pectolytic bacteria. Fig. 1 is a photograph of a plate of brilliant green gel showing isolated depressed colonies. The individual craters produced in either medium are quite distinct.

The results of several experiments indicated that these media did not require sterilization. However, the occluded air was not adequately released when sterilization was omitted.

Effect of the selective gels on the growth of molds and yeasts. Cultures of 21 molds and 17 yeasts were grown on the brilliant green bile and crystal violet gels in both the presence and absence of actidione. The inoculated media were incubated 96 hr at 20°C and examined for growth. These results are recorded in Table 1. A number of molds were able to grow on either medium in the absence of actidione and produce depressions indicative of pectolysis. However, actidione reduced the growth of these cultures so they were not able to produce depressions in 96 hr at 20°C. The reduction in growth and activity of the pectolytic molds is particularly desirable since they are commonly encountered as air-borne contaminants. It was also observed that actidione was more effective in inhibiting the growth of yeasts than that of molds. As a result, actidione was routinely added (10 mg/L) to either medium.

Fig. 1. Plate showing pectolysis by isolated colonies.

Effect of the selective gels on the growth of bacteria. An experiment similar to that described for molds and yeasts was conducted with 76 cultures of bacteria. The obligately anaerobic bacteria of the genus *Clostridium* were inoculated into deep tubes of gel with a Pasteur pipette. The surface of the gel was then stratified with sterile "vaspar" (1:1 w/w Parowax and Vaseline) before incubation. The inoculated media, both with and without actidione, were incubated 72 hr at 30°C before being examined for growth and pectolytic activity. The results are shown in Table 2.

It will be noted that most of the gram-negative bacteria tested grew moderately to profusely on either selective gel. It was also clear that, within the limits of this experiment, the two media were of nearly the same productivity. However, it should be emphasized that the *Acetobacter* species have fastidious growth requirements and would not be expected to grow under the conditions provided by these media.

It is possible that the slight growth observed with many of the cultures resulted from large numbers of cells transferred during inoculation. When a slight amount of growth was observed under these conditions it was invariably concentrated in the area at the beginning of the streak.

Effect of pH on utility of the selective gels. It was desirable to determine the effect of pH on the usefulness of the two selective gels because it is known that crude and partially purified bacterial "polygalacturonases" are most active *in vitro* at pH values between about 7.5 and 9.4.

Several experiments were run to determine the optimum pH over the range of pH 5.2 to 8.5 after sterilization of the media. The technique used was to make up the media and adjust pH to the desired levels. After autoclaving the pH values were determined again. The 19 test organisms were inoculated onto the surface of these test media by the giant-colony technique. The plates were incubated at 30°C and observed daily. The results indicated that the optimum pH was about 7.0 for growth and formation of the depressions indicative of pectol-

Table 2. Effect of selective polypectate media on growth of bacteria.[a]

Gram-negative bacteria	Brilliant green bile	Crystal violet	Brilliant green bile	Crystal violet
			Plus 10 mg/L actidione	
Acetobacter (10 cultures, 8 species)	—	—	—	—
Achromobacter parvulus	—	—	—	—
Achromobacter sp.			—	—
Aerobacter aerogenes	xx	xx	xxx	xxx
Aerobacter aerogenes	x	x	xxx	xxx
Aeromonas hydrophila	x	xx	x	xx
Alcaligenes sp.			xxx	xxx
Alcaligenes sp.	xx	xx	xxx	xxx
Arthrobacter atrocyaneus	—	x	x	x
Erwinia carotovora	xxxD	xxxD	—	xD
Erwinia chrysanthemi	xxxD	xxD	xD	xD
Escherichia coli	xx	xx	xx	xx
Escherichia coli	xx	xx	xxx	xxx
Escherichia coli	xx	xx	xxx	xxx
Escherichia freundii	xx	xx	xx	xx
Proteus vulgaris	xx	xx	xx	xx
Pseudomonas aeruginosa	xxx	xx	xx	xx
Pseudomonas aeruginosa	xx	xx	xx	xx
Pseudomonas mephitica	xx	xx	xxx	xxx
Pseudomonas mephitica	xxx	xxx	xx	xx
Pseudomonas putrefaciens	—	xx		
Pseudomonas putrefaciens	—	—	—	—
Pseudomonas sp.	xx	xx	xxx	xxx
Serratia marcescens	xxx	xxx	xxx	xx
Serratia marcescens	xxx	xxx	xxx	xxx
Vibrio sp.	—	—	—	—
Bacillus brevis			—	—
Bacillus coagulans	—	x		
Bacillus laterosporus	—	x	—	—
Bacillus laterosporus	—	x	—	—
Bacillus macerans	—	x	—	—
Bacillus polymyxa	x	x	x	—
Bacillus polymyxa	—	xx	—	—
Bacillus pumilus	—	x	—	—
Bacillus pumilus	x	x	—	—
Bacillus subtilis	—	—	x	x
Bacillus subtilis	—	—	—	—
Bacillus subtilis	—	x	—	—
Bacillus subtilis	—	—	—	—
Bacillus subtilis	—	—	—	—
Bacillus subtilis	—	—	—	—
Clostridium (14 cultures, 7 species)			—	—
Micrococcus lysodeikticus			x	x
Micrococcus pyogenes aureus			—	—
Micrococcus pyogenes albus			x	x
Sarcina lutea	x	x	x	x
Staphylococcus aureus	—	—	—	—
Staphylococcus aureus			—	—
Streptococcus faecalis	—	—		
Streptococcus lactis	—	x	—	—
Streptococcus lactis	—	—	—	—
Streptococcus sp.	x	—	—	—

[a] — no growth; x growth limited to area at beginning of streak; xx moderate growth; xxx profuse growth; D depressions.

ysis. For practical reasons, a pH at or near neutrality is best. Alkaline media become soft whereas acid media become stiff and unworkable.

The effect of sodium hexametaphosphate on utility of the selective gels. The formula for polypectate media, described first by Baier and Manchester (1943) and later by Vaughn *et al.* (1954, 1957), contained food-grade sodium hexametaphosphate (Calgon). The action of Calgon is to sequester calcium and other polyvalent ions. It was thought that elimination of this sequestering agent from the formula might strengthen the gel by salt bond formation. Besides, as already mentioned, calcium ions are required by some of the bacterial pectolytic enzymes.

Experiments were conducted with and without added Calgon as compared to media without Calgon but containing calcium chloride. The results indicated that, even without a precise method for determining gel strength, the media with Calgon were noticeably softer than the other media, so Calgon was omitted from the formula and calcium chloride was always added to ensure firmer gels.

Productivity of the selective gel media. As indicated in Tables 1 and 2, the two gel media were effective in preventing the growth of nearly all of the test organisms except the gram-negative bacteria. It was apparent, though, that there was some difference in the productivity of the two selective media, so a series of experiments were made to determine quantitative differences between the productivity of the two media as compared with the plate-count and other standard bacteriological media.

Total plate-count agar was used as the reference medium (American Public Health Association, 1958). Levin's eosine methylene blue (EMB) agar was tested because it is commonly used for detection of the gram-negative coliform bacteria. These media were compared in productivity with the two polypectate gels. A number of pure cultures of partially identified gram-negative pectolytic bacteria and two known cultures of *Pseudomonas aeruginosa* were used as test organisms. These cultures were grown 8–24 hr in nutrient broth at 30°C on a rotary shaker. Then the "total" count per ml was determined. Dilutions were made in the usual manner, and duplicate 0.1-ml portions were used as inocula for the spread-plate technique. The inoculated plates were incubated at 30°C, and counts were made after 12, 24, and occasionally 48 hr. The results are shown in Table 3.

The count from the total plate-count agar medium was assumed to represent 100% of the viable population in the culture. The counts obtained on the selective media were then compared with the total plate count by calculating the percentage of the populations that grew in comparison with those on the standard plate-count agar.

Table 3. Productivity of the selective polypectate media as compared with EMB agar and total plate-count agar.

Culture no.	Percentage recovery of viable cells			
	Plate-count agar	EMB agar	Crystal violet	Brilliant green bile
24 *Aerobacter*	100[a]	145	149	44
34 *Achromobacter*	100	0	119	7
38 *Paracolobactrum*	100	37	39	2
46 *Aeromonas*	100	41	55	36
62 *Aerobacter*	100	106	126	80
159 *Aerobacter*	100	95	103	62
160 *Aeromonas*	100	0	100	0
164 *Aerobacter*	100	67	115	42
164 *Aerobacter*	100	112	92	20
164 *Aerobacter*	100	102	82	60
164 *Aerobacter*	100	100	108	55
175 *Aeromonas*	100	43	75	28
177 *Escherichia*	100[b]	39	37	0
177 *Escherichia*	100[b]	97	86	0
178 *Achromobacter*	100	86	98	0
1 *Pseudomonas aeruginosa*	100[b]	228	151	87
1 *Pseudomonas aeruginosa*	100	167	100	73
2 *Pseudomonas aeruginosa*	100[b]	55	77	0
Average	100	84.4	95.1	33.1

[a] The count was assumed to be 100% of the viable count of each culture tested.
[b] Nutrient agar was substituted.

The brilliant green bile gel was not as productive as its crystal violet counterpart, and was the most inhibitory of the three media. It may also be concluded that the crystal violet gel is at least as productive as the EMB agar since the average count in the crystal violet gel medium is almost the same as the total plate-count agar under the conditions described here.

Recovery of pectolytic gram-negative bacteria from in vitro mixtures of two cultures. Several experiments were conducted to determine the recovery of pectolytic gram-negative bacteria from intentional mixtures of two cultures. The two cultures were grown in yeast extract-glucose broth in individual side-arm Erlenmeyer flasks on a shaker at 30°C. After 4–8 hours the cultures were diluted with sterile distilled water to the same optical density (0.2). The cultures were then mixed 1:3, 1:1, and 3:1 and plated with the spread-plate technique on total plate-count agar and the two polypectate gels. The plates were counted for

Table 4. Recovery of gram-negative pectolytic organisms from a mixture of gram-negative bacteria.[a]

Ratio of culture No. 62 *Aerobacter* to *Ps. aeruginosa*	Media	Anticipated count × 10^6 [b]	Actual count × 10^6
3:1	PC		76
	CV	57D 19	68D 17
	BG		48D 2
1:1	PC		283
	CV	142D 142	136D 87
	BG		74D 21
1:3	PC		220
	CV	66D 126	66D 126
	BG		26D 21

[a] PC total plate-count agar; CV crystal violet polypectate; BG brilliant green bile polypectate; D depressions.

[b] Anticipated count based on total plate-count agar total count.

total numbers, and on the polypectate gels, for pectolytic and non-pectolytic colonies.

The results are shown in Table 4. The "anticipated" count was calculated on the basis of "total" count on the total plate-count agar. It was observed that the actual count was the same as that expected, and the variation could be interpreted as an inherent error in the plating method. The brilliant green bile medium permitted less growth than the crystal violet, confirming the results above. *Ps. aeruginosa* was inhibited to some extent on the polypectate media, as shown by the decrease in the numbers of non-pectolytic colonies. These results show that these pectolytic gels allow nearly quantitative recovery of pectolytic organisms from mixtures of gram-negative bacteria.

A second set of experiments were run to examine the effectiveness of these media in preventing growth of *Bacillus* species and allowing quantitative recovery of pectolytic gram-negative bacteria. The procedure differed from that described above in that the cultures were grown on nutrient broth, and counted with a Petroff-Hauser bacteria chamber before plating, and the plating procedure was modified.

The experiments were made with cultures mixed 3:1, 3:2, 1:1, and 1000:1 of *Bacillus subtilis* to the unidentified culture No. 62, an *Aerobacter*. Unmixed cultures were plated on nutrient agar and the polypectate gels to determine the "total" count, and the mixtures were plated on the two selective gels.

The results (Table 5) indicate that the plate counts were as expected when calculated on the basis of the polypectate gels as total count. Therefore, the selective polypectate media apparently are effective in preventing the growth of *B. subtilis* when it is mixed in equal portions or in massive numbers with respect to the gram-negative pectolytic test bacteria. All of the colonies on the polypectate media were similar and typical of the pectolytic gram-negative culture.

On the basis of these *in vitro* experiments, several conclusions appear warranted. Pectolytic gram-negative bacteria can be separated from mixed cultures and enumerated on the selective gels. The ratio of populations of pectolytic gram-negative bacteria to non-pectolytic bacteria or pectolytic gram-positive bacteria had no effect on the selectivity of the media. The crystal violet polypectate gel was consistently more productive than the brilliant green bile medium.

Recovery of pectolytic gram-negative bacteria from mixed cultures from natural sources. An important test of the utility of the selective gel media is, naturally, an evaluation of their usefulness for recovery of pectolytic gram-negative bacteria from mixed cultures from various natural sources. Pectolytic gram-negative bacteria have been isolated from the following sources on both brilliant green and crystal violet gels: fermenting cucumber, olive, and pepper brines; soft, rotten carrots, let-

Table 5. Recovery of gram-negative pectolytic organisms in a mixture of *Bacillus subtilis*.

Ratio of culture No. 62 to *B. subtilis*	Media [a]	Anticipated count × 10^6 [b]	Actual count × 10^6
0:1	Nut	144[c]	40
	CV		0
	BG		0
1:0	Nut	272[c]	226
	CV		283D
	BG		93D
1:3	CV	71	70D
	BG	23	15.9D[d]
2:3	CV	113	148D
	BG	37	33D
1:1	CV	142	136D
	BG	37	38D
0:1	Nut	73.8[c]	12.7
	CV		0
	BG		0
1:0	Nut	383[c]	229
	CV		208D
	BG		88D
1:1000	CV	10.4	10.3D
	BG	4.4	3.7D

[a] Nut nutrient agar; CV crystal violet polypectate; BG brilliant green bile polypectate.

[b] Anticipated count based on polypectate gels total count.

[c] Microscopic count.

[d] Depressions.

tuce, and potatoes; softened olives undergoing processing; well water from an olive processing plant; soil samples from olive groves and cucumber fields; unblanched dehydrated vegetables; aseptically shelled almond and English walnut meats; and male human feces.

In these studies the only cultures producing crateriform depressions in either gel were gram-negative rods. Colonies of mold or yeast that developed were small, isolated, and visually recognizable. It is believed that either gel will be useful for further studies on the ecology of the pectolytic gram-negative bacteria but the crystal violet medium is recommended because it is less inhibitory, at least under the conditions described here.

During this investigation several observations were made that have a bearing on the utility of these two gels. Some organisms have a tendency to absorb the crystal violet from the medium, thus forming a blue colony. Other organisms do not absorb the dye, so the colonies remain colorless. This affords a means of differentiating these types of colonies on a plate of crystal violet gel with mixed cultures.

On the brilliant green medium the colonies do not absorb the dye, and thus appear white against a green background. In a few instances the background turns yellow. This latter color difference facilitates the counting of colonies. In contrast, the colonies that absorb the crystal violet are difficult to observe before they form depressions in the medium. This problem may be solved either by pouring the medium in a thinner layer to reduce its opacity, or by counting the colonies after they have formed depressions in the medium.

When moisture was present on the surface of the brilliant green gel, the colonies occasionally spread. The problem of spreading was not encountered with the crystal violet gel. Reduction of the surface tension by the bile in the brilliant green medium might explain this difference.

The appearance of depressions on the surface was generally 6–12 hr later with the crystal violet gel than with the brilliant green medium. The times were respectively after 24 hr and 12–18 hr of incubation.

Pectolytic enzymes produced by cultures isolated on the two gels. *In vitro* demonstration of pectolysis by enzymes produced by presumably pectolytic gram-negative cultures of bacteria recovered from either of the two gels would generally be considered as final proof of their utility as differential media. Pectolytic enzymes known to be produced by bacteria include pectin esterase (PE), "polygalacturonase" (PG), and pectic acid eliminase (PAE).

Pectin esterase production was demonstrated by a modification of the method of McComb and McCready (1958). Instead of using a crude enzyme solution on the plates, these organisms were grown on nutrient agar containing 1% pectin n.f. at pH 7.0. After 48 hours or more of growth the test reagents were added. A clear zone surrounding the colony is indicative of PE activity. "Polygalacturonase" production was detected by the "cup plate" assay method described by Nagel and Vaughn (1961a). The cultures were grown 48 hr or longer at 30°C in nutrient broth containing 0.5% sodium polypectate. The cells were removed by centrifugation, and 0.05 ml of the supernatant of each culture was tested in a "cup" for PG activity on 1% pectic acid contained in 1% agar at pH 7.0.

PAE (Nagel and Vaughn, 1961b), similar in some respects to the pectin trans-eliminase of Albersheim *et al.* (1960), was demonstrated by first growing the 19 gram-negative control cultures in a medium containing 0.5% pectic acid, 0.2% Bacto yeast extract, and buffered at pH 7.0 with 1% Tris buffer. After incubation for 48 hours at 30°C on a rotary shaker the cultures were centrifuged. The supernatant was dialyzed, then tested for PAE activity in a Beckman DU spectrophotometer at a wavelength of 235 mμ to measure the increase in optical density indicative of the formation of unsaturated bonds during degradation of the pectic acid. The reaction mixture contained 1.5 ml of 0.3*M* Tris buffer at pH 8.0, 1.0 ml of 1% pectic acid at pH 8.0, 0.3 ml of 0.01*M* $CaCl_2$, and 0.2 ml of the dialyzed crude enzyme supernatant. The reaction time varied from approximately 8 to 30 min at room temperature.

All of the control cultures produced an active PAE and non-specific PG, and were able to de-esterify pectin. Therefore, it may be concluded that the two polypectate gels have good differential value.

ACKNOWLEDGMENT

Financial assistance from the California Olive Advisory Board is gratefully acknowledged.

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Factor Analysis Applied to Paired Preferences Among Four Grape Juices

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(Manuscript received April 24, 1961)

SUMMARY

Four grape juices differing in amount of added citric acid were examined by paired comparison by 72 tasters. Some judges preferred sweet and some preferred acid juices, causing pronounced and progressive changes in the frequency distributions of the preference ratings. The correlation coefficient between variances and acid differences was 0.98. The direction and degree of preference was much more consistent for larger differences in acid. Mathematical models developed by other authors for analysis of paired comparison data are of doubtful validity or awkward in application to the present data.

INTRODUCTION

The mathematical models detailed by Dykstra (1960), Scheffé (1952), and Terry *et al.* (1952) for analysis of data for paired comparison cannot be easily used when the distribution of judges' scores are bimodal with different variances. The method described by Pangborn and Nickerson (1959) could be applied, but only with certain awkwardness.

Because the tasters in the present experiment separated into two distinct groups with regard to direction and degree of preference, the factor analysis exemplified by Baker *et al.* (1955) and explained by Guilford (1954) was used.

EXPERIMENTAL PROCEDURE

Juice from White Riesling grapes, close-filtered twice, was a light yellow and quite brilliant color. The reducing sugar content of the grape juice was 23.5 g/100 ml. The amounts of citric acid added to the original juice to produce the samples designated A, B, C, and D were respectively 0, .091, .274, and .548 g/100 ml. To prevent precipitation of potassium acid tartarate the acid additions were made each day just before the tests. The total acids, expressed as tartaric acid/100 ml, for the three days were:

Juice	1st	2nd	3rd	Av.	Expected [a]
A	0.38	0.39	0.39	0.386	0.386
B	0.46	0.47	0.53	0.486	0.503
C	0.73	0.69	0.69	0.703	0.708
D	0.99	1.02	1.02	1.005	1.030

A panel of 72 tasters gave direction of preference and degree of preference between each of the 6 possible pairs. Tastings were on 2 consecutive days immediately succeeding a training run on one day. The details of presenting the pairs for comparison followed procedures worked out over several years in numerous similar experiments. In particular, the trials were made in such a way as to balance out order in pairs and the effect of days. The tasters were staff members and students. The data consist of paired comparisons of each of the six possible pairs by each of 72 testers. The first day's data (which are, as far as method of presentation is concerned, exactly like the last day's data) are omitted since the first day was designed to be simply a training run. These data are presented in Table 1. The direction of preference is indicated by minus and plus signs, and the degree of preference by the scale values 0, 1, 2, 3. Thus the complete scale for comparisons of pairs is −3, −2, −1, 0, 1, 2, 3.

[a] A (original juice) plus added amount of citric acid converted to tartaric acid equals the expected amount.

Table 1. Preference scores for 72 tasters for 6 paired comparisons. The numbers indicate the preference for the first member of each pair over the second.

	AB	BC	AC	CD	BD	AD
1	1	2	2	3	3	3
2	1	1	3	3	3	3
3	−2	1	−2	1	2	3
4	−2	1	3	0	−2	2
5	1	3	3	0	3	3
6	2	1	1	1	2	2
7	−1	−1	−3	3	−3	−3
8	2	3	2	2	3	3
9	1	2	2	2	3	3
10	0	1	2	2	3	3
11	−2	−2	−2	3	−2	−2
12	−1	−2	−3	−2	−3	−3
13	−2	−2	−3	3	3	3
14	−1	−2	−2	−2	−1	−2
15	1	−2	−2	−1	−2	−3
16	−1	1	−2	2	2	3
17	−2	0	−2	−3	−3	−3
18	0	−2	2	3	3	3
19	−2	−1	−2	−2	−2	−2
20	2	−1	3	3	2	3
21	1	1	−2	−1	−2	−2
22	−1	−2	−1	3	3	3
23	−1	−2	−2	2	−2	−3
24	1	1	2	2	2	2
25	−1	2	−2	−2	3	−3
26	−2	−3	−3	−3	−3	−3
27	1	1	−1	1	1	3
28	−1	−1	−3	−1	−3	−3
29	−1	1	1	2	2	2
30	1	−1	−2	−2	−3	−2
31	−1	−2	−2	−2	−2	−2
32	−1	−2	2	2	3	3
33	−1	2	3	2	−3	3
34	−1	−1	−2	−3	−3	−3
35	−2	−2	−2	−1	−1	−2
36	−1	−2	−2	−1	3	3
37	−2	−2	−2	−1	−1	−1
38	−1	2	−2	2	3	3
39	1	2	2	3	3	3
40	2	−1	−2	−1	−1	−2
41	−1	−1	−2	−3	−2	−3
42	1	−2	−3	2	3	3
43	−2	0	0	0	3	−3
44	−2	−2	−3	−3	−3	−3
45	0	−2	2	−1	−2	−1
46	−1	−1	−3	−2	−2	−3
47	−1	−1	−2	−2	−3	−2
48	1	−2	−1	−1	−1	−3
49	−1	−2	−3	3	3	3
50	−2	−2	−3	2	−3	−2
51	−2	−2	−3	−2	−3	−3
52	−1	−1	−2	−3	−3	−3
53	0	2	1	1	3	3
54	−1	1	2	2	2	−2
55	0	−1	−2	−2	−1	−2
56	−1	−1	−1	2	−1	−2
57	−1	1	−1	−1	−2	−1
58	−1	−2	−1	−2	−2	−2
59	−3	−2	−3	−2	3	3
60	2	2	3	−2	1	1
61	2	−2	−2	−1	−2	−1
62	−2	2	−2	−3	−3	−3
63	−2	−2	−2	−2	−2	−2
64	1	0	1	2	−2	3
65	2	−1	2	−2	−3	−2
66	3	−1	3	1	2	−2
67	2	2	−3	3	3	−2
68	−1	−2	−2	2	−2	−3
69	−1	0	0	−1	−1	−1
70	−1	−1	−2	−2	−2	−3
71	1	2	3	3	3	3
72	0	−2	−2	−2	−3	−2
Mean	−.38	−.43	−.71	.08	−.12	−.26
σ^2	1.98	2.67	4.51	4.50	6.22	6.76
Sum absolute values	93	111	151	140	171	181
Neg. values	60	71	101	67	90	100
Pos. values	33	40	50	73	81	81

ANALYSIS AND DISCUSSION OF DATA

Scatter diagrams were made of the joint ratings of the tasters on pairs of different juices as detailed in Table 1. It appeared that a conventional factor analysis was proper. Therefore the correlation matrix given in Table 2 was computed. The diagonal elements were assumed to be equal to the greatest coefficient in the corresponding column.

Two factors were extracted as indicated in Table 3: one we have called F_1, the "sugar" factor, and one called F_2, the "acid" factor. We rotated the original factors into F_1' and F_2' in such a way as to make the F_1' loading on the pair AB as large as possible. We note that when this is done, the F_2' loadings on AB, BC, and AC are zero. However, the F_2' loadings on CD, BD, and AD are about equal to the F_1' loadings but opposite in sign. Thus, we have two very close clusters of pairs of 3 each: one on the positive F_2' axis, and the other on a line that makes a minus 45° angle with the positive F_2' axis.

h^2 (communality) measures the ability of the tasters to prefer between the pairs irrespective of their direction of preference, while $1-h^2$ measures the inability of the tasters to prefer. It is noted that h^2 ranges from 0.41 to 0.78. On the basis of the communalities, the 6 pairs can be ordered in a rational way. These findings are in accord with the discussions by Baker *et al.* (1954, 1958, 1960) and Mrak *et al.* (1959).

The detailed frequency distributions in Table 4 indicate the very pronounced changes in the distributions of preferences as successive amounts of citric acid were added. Progressive and extensive changes take place in these distributions as we proceed from pair AB to pair AD. The variances of the 72 tasters increase from 1.98 for the pair AB to 6.76 for AD. It is apparent that as "sugar" juices are compared with "acid" juices, some people much prefer the sugar and many others much prefer the acid.

If we consider the relation between amount of citric acid added and $100h^2$ of Table 1, we get the results in Table 5. The correlation coefficient is 0.961, indicating a

Table 2. Correlation matrix for the factor analysis for the data of Table 1.

	AB	BC	AC	CD	BD	AD
AB	(0.50)[a]	0.35	0.50	0.27	0.30	0.46
BC	0.35	(0.54)	0.54	0.30	0.44	0.43
AC	0.50	0.54	(0.54)	0.41	0.43	0.54
CD	0.27	0.30	0.41	(0.64)	0.62	0.64
BD	0.30	0.44	0.43	0.62	(0.75)	0.75
AD	0.46	0.43	0.54	0.64	0.75	(0.75)

[a] Communalities. See E. C. Baker *et al.* 1955.

Table 3. Factor loadings on two factors (F_1, sweetness; F_2, acidity) for the six pairs of Table 1 rotated to make the loading of F_1' on pair AB a maximum.

Pair	F_1	F_2	λ-matrix		F_1'	F_2'	h^2[a]
AB	.57	.29	.909	−.417	.64	.03	.41
BC	.62	.28	.417	.909	.68	.00	.46
AC	.70	.32			.77	.00	.59
CD	.69	−.32			.56	−.58	.65
BD	.78	−.34			.57	−.63	.72
AD	.85	−.23			.68	−.56	.78

[a] h^2 is the communality or sum of squares of the factor loadings.

Table 4. Frequency distributions of the preference ratings of 72 tasters for the 6 comparisons of Table 1. The preference scale is in the direction A to D.

Preference						
−3	1	1	13	7	16	19
−2	15	26	28	17	17	19
−1	27	16	6	12	8	5
0	6	4	2	3	0	0
1	14	12	4	5	2	1
2	8	11	11	16	8	4
3	1	2	8	12	21	24
Total	72	72	72	72	72	72

Table 5. Regression of discriminability ($100h^2$ of Table 3) on the difference in citric acid in grams/ml for the 6 pairs of Table 1.

Pair	(x = acid difference)	(y = 100 h^2) Observed	Calculated
AB	0.091	41	42.69
BC	.183	46	50.22
AC	.274	59	57.67
CD	.274	65	57.67
BD	.457	72	72.65
AD	.548	78	80.10
Total	1.827	361	
SS deviations	0.144753	1050.8	

Least-squares regression line: $y = 81.850\,x + 35.243$
Correlation coefficient: $r = 0.961$

Table 6. Regression of the sums of the absolute values of the preference scores on the difference in citric acid added, in grams/ml, for the 6 pairs of Table 1.

Pair	(x = acid difference)	(y = sum of absolute values) Observed	Calculated
AB	.091	93	99.94
BC	.183	111	117.67
AC	.274	151	135.21
CD	.274	140	135.21
BC	.457	171	170.53
AD	.548	181	188.01
Total	1.827	847	
SS deviations	.144753	5,804.8	

Least-squares regression line: $y = 192.970\,x + 82.407$
Correlation coefficient: $r = 0.964$

nearly perfect relation between $100h^2$ and the difference between the amounts of citric acid added to the separate juices of a pair.

Another possible measure of the strength of preference without regard to the direction of the preference is the sum of the absolute values of the preference ratings for each pair. Table 6 shows the relation between the sums of the absolute values of the preference scores and the acid difference between pairs. The correlation coefficient is about the same as before, $r = 0.964$, showing that these variables are very closely related.

ACKNOWLEDGMENTS

We thank C. S. Ough for help in preparing and standardizing the juices, and Marion Simone and C. S. Ough for assistance in collecting the data.

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Taste Interrelationships. II. Suprathreshold Solutions of Sucrose and Citric Acid

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(Manuscript received May 26, 1961)

The effect of citric acid on the sweetness of sucrose solutions was determined by a highly trained panel using variations of two basic methods: single stimulus and paired stimuli. In the former presentation, evaluation of samples singly or in a series of 4 did not alter responses significantly. The binary method, requiring judges to indicate the direction and the degree to which members of a pair differed in sweetness, was slightly more sensitive than the single presentation, especially when one member of the pair within a set was kept constant. The following conclusions were drawn from all methods: (a) Citric acid, at concentrations ranging from 0.007 to 0.073%, depressed the sweetness of 0.5–20.0% sucrose. (b) The masking of sweetness by acid was greater at lower than at higher sucrose concentrations.

INTRODUCTION

Methods of measuring taste interactions of sapid solutions vary considerably, depending on the purpose of the study and the preferences of the investigator. The literature contains conflicting results obtained with diverse methods. In an early study, Kiesow (1894) applied solutions directly on the tongue and observed the effect of a subthreshold concentration of one compound on the threshold of a second compound. Salty and sour, and salty and sweet exhibited simultaneous masking, but sweet and sour solutions affected each other in an inconsistent manner so that no conclusions could be drawn. Working with sucrose, NaCl, quinine hydrochloride, and HCl, Heymans (1899) found that supraliminal concentrations of one substance always raised the threshold for a second compound. Only one subject was used in that investigation. Werner (1933) matched the tastes of inorganic salts at fixed concentrations against substances representing the 4 fundamental tastes. He found that diluting a salt solution and its taste-matched sugar solution to the same degree did not result in maintenance of equivalent tastes, and concluded that "interaction relationships between taste stimuli are specific to the level stimulus intensity at which they occur."

Bujas (1934) and Hahn (1936) used the "Geschmackslupe" (U-tube) to stimulate one area of the tongue with sucrose solutions while simultaneously determining the threshold for NaCl on another part of the tongue. Dilute sucrose solutions lowered the NaCl threshold, and concentrated sucrose solutions raised it. Similar results were reported in a later publication (Hahn and Ulrich, 1948).

The "sensitivity threshold," i.e., the lowest concentration distinguishable from water, was used by Cragg (1937) to establish the effect of NaCl and sucrose on the sourness of HCl. The addition of 18.7% sugar did not change the HCl threshold but the addition of 2.9% sugar reduced the sourness of 0.005*N* HCl by 15%. Equi-sweet saccharin solutions produced the same effects as sucrose.

In one of the most comprehensive studies reported, Fabian and Blum (1943) selected a matching method in which a suprathreshold concentration of one compound plus a threshold level of a second compound was matched to a series of 5 solutions of increasing concentration of the suprathreshold substance. They reported that hydrochloric and acetic acids decreased the sweetness of dextrose but had no effect on the sweetness of sucrose. Lactic, malic, citric, and tartaric acids in-

creased the sweetness of sucrose but decreased the sweetness of fructose. In addition, all sugars decreased the sourness of all acids, though to varying degrees. Using the same matching methodology, Pangborn (1960) found that sucrose and citric acid had a masking effect on each other.

Anderson (1950) used a single-sample presentation of sucrose, sodium chloride, quinine hydrochloride, and tartaric acid, all at threshold levels. In tap water, sucrose thresholds for the two subjects used were 0.029*M* and 0.012*M* whereas in the presence of threshold levels of tartaric acid, the respective sucrose thresholds rose to 0.038*M* and 0.016*M*. The corresponding respective values for tartaric acid were 0.0012*M* and 0.00035*M* in tap water and 0.0010*M* and 0.00038*M* in threshold concentrations of sucrose.

In a study of the taste interactions of sucrose and sodium chloride ranging from threshold to solubility limits, Beebe-Center *et al.* (1959) employed the "up and down" method, i.e., tasters indicated whether the second sample within a pair was more or less sweet than the first. When the compound was judged less sweet than the least concentrated standard of the scale, or sweeter than the most concentrated, the same standard was used again in the next comparison. The median was used as the measure of central tendency. The principal effect was one of mutual masking. A single-stimulus method with a nine-point scale of successive integers from 1 (no taste) to 9 (extreme intensity) was described by Kamen *et al.* (1960), who concluded that suprathreshold levels of citric acid generally increased the sweetness of suprathreshold concentrations of sucrose, whereas sucrose reduced the sourness of citric acid.

It is difficult to make a critical comparison of the precision of the methods discussed above since the results were influenced by many other experimental variables, i.e.,

SCORE CARD FOR SINGLE-SAMPLE PRESENTATION SET_____

NAME_____ DATE_____

DIRECTIONS: CIRCLE THE VERTICAL LINE CORRESPONDING TO YOUR OPINION OF THE TASTE INTENSITY OF SWEETNESS FOR EACH SAMPLE SEPARATELY.

SAMPLE NO.

	None	Slight	Moderate	Large	Extreme
_____					

SCORE CARD FOR PAIRED PRESENTATION

SET_____

NAME_____ DATE_____

DIRECTIONS: CIRCLE THE SWEETER SAMPLE WITHIN EACH PAIR, THEN INDICATE THE DEGREE OF DIFFERENCE IN SWEETNESS WITHIN PAIRS.

SAMPLES		SWEETNESS DIFFERENCE WITHIN EACH PAIR			
		SLIGHT	MODERATE	LARGE	EXTREME

Fig. 1. Score cards used for single-sample and paired presentations.

number of subjects, training of judges, amount of replication, temperature of the solutions, tap water vs. distilled water, buffered vs. unbuffered solutions, purity of the compounds, and the levels compared.

The present paper is concerned with evaluation of the taste reactions of a highly trained panel to mixtures of suprathreshold concentrations of sucrose and citric acid using variations of single and paired presentation.

METHODS AND MATERIALS

Selection and training of the taste panel. Nine judges, 4 men and 5 women 25–45 years old, were selected from 23 individuals on the basis of their ability to distinguish small differences in concentration between paired solutions of sucrose and of citric acid. Solutions were freshly prepared each day and served at room temperature in 40-ml portions in coded beakers presented in randomized order. Freshly distilled water was provided for oral rinsing at the judge's discretion. All evaluations were made between 11 and 12 A.M. Monday through Friday, in individual partitioned booths. Participants were informed of their accuracy after each test session.

Methods. *Single-sample.* The score card described by Kamen *et al.* (1960) was used; a nine-point rating scale of taste intensity, with alternate points "anchored" by the descriptive terms "none, slight, moderate, strong, extreme." Later, the points were assigned successive integers from 1 to 9 and analysis of variance applied to the scores. In Method I, judges received a tray containing 4 solutions (randomly selected from the 16 possible combinations) presented in randomized order. The sweetness intensity of each solution was recorded separately; however, comments indicated that the samples were influencing each other, i.e., the sweetness intensity of the first sample affected the apparent sweetness of the subsequent samples within a set. To offset this contrast effect, the investigation was repeated using Method II: one solution only was evaluated per judge per day. The experimental design was repeated 4 times/judge for Method I and once for Method II. Analysis of variance was applied to individual intensity scores. Only replication I in Method I was analyzed against Method II.

Paired samples. After completion of the above study, paired presentation was tested. Each judge received a tray containing 8 pairs for Series I, and 12 pairs for Series II. Participants circled the number of the sweeter sample and indicated the degree of difference in sweetness intensity within each pair by checking a four-point scale labeled "slight, moderate, large, extreme." This was an adaptation of a preference method described by Scheffé (1952). Upon the recommendation of Gridgeman (1960, 1961), the central (zero) point was eliminated so as to force a decision.

The paired-stimulus method was repeated in Series II, using a different range of sucrose and citric acid. The entire experimental design was repeated 4 times/judge for Series I, and 3 times/judge for Series II. For statistical treatment of the data, negative and positive numbers were used to indicate the direction and sweetness intensity of the response, as illustrated below:

A (no acid) > B (0.005% acid)
−7, −5, −3, −1

A (no acid) < B (0.005% acid)
+1, +3, +5, +7

Negative and positive responses were totaled for each pair, the weighted values plotted, then linear

Table 1. Concentrations used in the various presentation designs.

Single sample, methods I and II

% citric acid [b]	% sucrose [a]			
	0.50	1.80	5.80	20.0
0	A	B	C	D
0.007	E	F	G	H
0.023	I	J	K	L
0.073	M	N	O	P

Paired samples, series I

Concentrations same as in single samples, above.

Pairs:	AE	EI	IM
	BF	FJ	JN
	CG	GK	KO
	DH	HL	LP

Paired samples, series II. Constant stimuli

% citric acid [b]	% sucrose [a]				
	1.0	2.0	5.0	10.0	20.0
0	A	B	C	D	E
0.005	F	G	H	I	J
0.010	K	L	M	N	O
0.020	P	Q	R	S	T
0.040	U	V	W	X	Y
Pairs:	AF	BG	CH	DI	EJ
	AK	BL	CM	DN	EO
	AP	BQ	CR	DS	ET
	AU	BV	CW	DX	EY

[a] Baker analyzed.
[b] Baker and Adamson. Percentages have been corrected for the one molecule of water of crystallization.

regression determined so that the slopes of individual lines could be compared for Series I. For Series II, the weighted responses were submitted to analysis of variance to establish the degree to which citric acid influenced sweetness intensity.

Score cards for the single and paired presentations are shown in Fig. 1.

Taste solutions. The concentrations used in the various designs are shown in Table 1.

The paired design was repeated with the concentrations indicated in Series II when the panel complained of the extreme displeasure involved in continuous sampling of the 0.073% level of citric acid. Also, no sweetness at all was perceived in solutions containing 0.50% sucrose in the presence of 0.007, 0.023, or 0.073% acid.

RESULTS AND DISCUSSION

Single-sample presentation. Although the methods of presentation, singly or in series, had no significant effect on sweetness scores (Table 2), the plotted data show that the scoring range was narrower when samples were evaluated singly than when tasted in a series of 4 solutions (Fig. 2). In the latter method, the "contrast error" may have been influential, i.e., a tendency to undervalue or overvalue a given characteristic within a series (Guilford, 1954, p. 279). This tendency is indicated by the significant interaction between sucrose and method, and is readily seen by determining the difference in total scores within each method:

% sucrose	Method I (series)	Method II (singly)	Difference
0.50	64	78	−14
1.80	124	122	+ 2
5.80	208	199	+ 9
20.00	304	283	+21

Judges responded differentially to the two methods; the difference in total sweetness scores between methods ranged from −18 to +14 for individual judges. The interaction between methods and acid level was not significant. Application of analysis of variance to Method I, separately, showed that the variation due to replication (3 reps.) was not significant.

With both methods of presentation, increasing additions of citric acid generally decreased apparent sweetness intensity at each of the 4 sucrose levels. This observation, although in agreement with previous work in this laboratory (Pangborn, 1960), disagrees with the conclusions of Fabian and Blum (1943) and of Kamen *et al.* (1960), who reported that citric acid increased the sweetness of sucrose. In the present investigation, all of Kamen's experimental conditions were followed except for selection and training of judges and presentation of 8 stimuli. In Kamen's study, there were 700 untrained judges selected on the basis of availability; only some participated in more than one replication. Kamen's group feel that this method increases the efficiency of the design by avoiding loss of motivation through testing of all solutions at one sitting and that "training and using more rigorous methods of selecting *Os* is probably not worth the effort. Instead, using a larger number of *Os* is likely to compensate for somewhat decreased precision." This latter point had been verified by Kamen previously (1959). The present author does not subscribe to the use of untrained, untested judges in difference testing, because a judge's response is greatly modified by

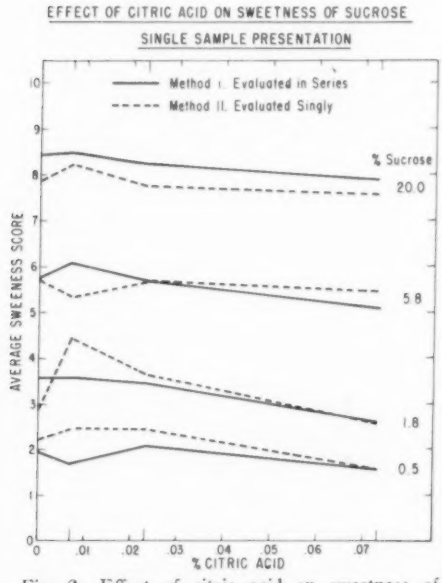

Fig. 2. Effect of citric acid on sweetness of sucrose single-sample presentation.

Table 2. Analysis of variance. Single-sample presentation. Method I, evaluated in series; Method II, evaluated singly.

Source of variation	Sums of squares	Degrees of freedom	Mean square	F ratio
Total	1928.319	287		
Sucrose (S)	1575.236	3	525.079	773.312***
Citric (C)	23.847	3	7.949	11.707***
Judges (J)	88.069	8	11.009	16.214***
Method (M)	1.125	1	1.125	1.657
S × C	10.680	9	1.187	1.748
S × J	35.764	24	1.490	2.194***
S × M	8.902	3	2.967	4.369**
C × J	20.153	24	0.840	1.237
C × M	1.625	3	0.542	0.798
M × J	26.500	8	3.313	4.879***
Remainder	136.391	201	0.679	

** Sig. at P = 0.01.
*** Sig. at P = 0.001.

training and familiarization with the tasting method, as illustrated by increased sensitivity to the 4 basic tastes with subsequent testing (Pangborn, 1959). Several investigators have reported improved sensory performance as a direct result of panel training (Bennett *et al.*, 1956; Boggs and Hanson, 1949).

When data from both methods were pooled, it was observed that every judge gave a higher total sweetness score to solutions containing 0.007% citric acid than to the unacidified control. This result is not readily explainable. Individual judges frequently commented that the lowest acid level imparted a degree of pleasantness to otherwise insipid sugar solutions. With this particular method, there is no way of determining quantitatively how much of the observed variation in scores was due to differences in palatability rather than taste intensity.

Paired presentation. In Series I of the paired presentation, samples differed in both sucrose and citric acid content. A simple tally of the selections of sweeter samples within pairs shows a decrease in apparent sweetness with increasing acid levels (Table 3). At lower sugar levels, the acid had a greater sweetness-depressing effect than at higher levels. When the data from Table 3 are weighted in terms of degree of sweetness difference between members of a pair, i.e., slight, moderate, large, extreme, a separate value is obtained for each pair, which is then plotted at each sucrose level in Fig. 3. The plot shows the same distribution as the data in Table 3: (a) 0.023% vs. 0.073%

Table 3. Effect of citric acid (percent) on sweetness in three paired presentations, series I (values represent response to sweetness within each pair).

	I		II		III		Totals	
% sucrose	0 *	0.007 *	0.007 *	0.023 *	0.023 *	0.073 *	Lower acid	Higher acid
0.50	25*	11	34***	2	31***	5	90***	18
1.80	32***	4	33***	3	33***	3	98***	10
5.80	24	12	25*	11	29***	7	78***	30
20.0	22	14	21	15	25*	11	68**	40
Totals	103***	41	113***	31	118***	26	334***	98

* Sig. at P = 0.05.
** Sig. at P = 0.01.
*** Sig. at P = 0.001.
* Percent citric acid.

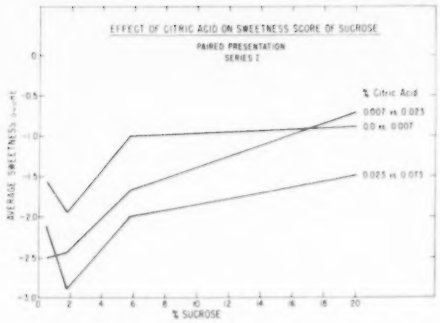

Fig. 3. Effect of citric acid on sweetness score of sucrose; paired presentation, series I.

citric acid gave greater differences than did the other two pairs; (b) sweetness masking was greater at lower than at higher concentrations of sucrose. The slopes of the three lines did not differ significantly as indicated by a statistical comparison of the b values. An unexplainable drop at the 1.8% sugar level was observed in two of the plots. Although the paired presentation data from Series I involved more complex calculation and interpretation than data obtained by single-sample presentation, the same general conclusion was observed: at the levels studied, citric acid decreased the apparent sweetness of sucrose.

A simplification of the paired methodology is incorporated into Series II, the method of constant stimulus, where one member of the pair within each set was constant. The simple tabulation in Table 4 verifies that a decreasing sweetness accompanied an increasing acid content. Once again, the masking effects were most pronounced at the lower sugar levels. At higher sugar levels, a mutual masking actually occurred and the acidified samples did not taste as sour. Plotting the weighted sweetness scores for each pair against acid content shows the degree to which the acid decreased sweetness (Fig. 4). Although the intermediate sugar levels follow a somewhat erratic downward progression, the extremes, 1.0 and 20.0% sucrose, substantiate the previous observation that higher levels are depressed less than lower levels. The author agrees with statements by Werner (1933), Cameron (1947), Kamen (1959), and others that perceived intensities of mixtures depend on the specific levels of each stimulus.

The individual values plotted in Fig. 4 readily lend themselves to analysis of variance (Table 5). Both sugar and acid had a significant effect on sweetness differences between pairs. A very highly significant variation was due to judge differences. The 3 replications did not differ, and two of the interactions involving judges were significant.

Analysis of variance is as simple to perform for the paired comparison data as for the single-sample scores. The former method requires that each judge replicate every set n times, so that the values designated as direction and magnitude of difference between a pair are based on the same number of evaluations. This requisite was not difficult to achieve, since only 5 days of testing (12 pairs/judge/session) were necessary to secure the data analyzed in Table 5.

Although the main advantage of the single stimulus is its simplicity of presentation and

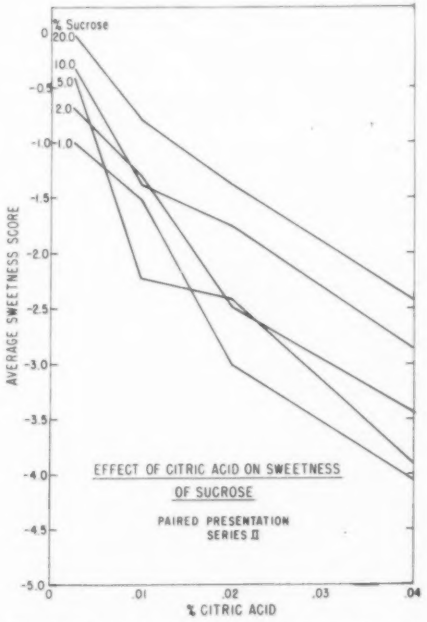

Fig. 4. Effect of citric acid on sweetness score of sucrose; paired presentation, series II.

Table 4. Effect of citric acid (percent) on sweetness in four paired presentations, series II (values represent response to sweetness within each pair).

	I		II		III		IV		Totals	
% sucrose	0 [a]	0.005 [a]	0 [a]	0.010 [a]	0 [a]	0.020 [a]	0 [a]	0.040 [a]	No acid	Added acid
1.00	21**	6	22**	5	27***	0	27***	0	97***	11
2.00	19	8	22**	5	24***	3	25***	2	90***	18
5.00	18	9	23***	4	24***	3	24***	3	89***	19
10.00	18	9	22**	5	19	8	21**	6	80***	28
20.00	14	13	16	11	19	8	21**	6	70***	38
Totals	90***	45	105***	30	113***	22	118***	17	426***	114

** Sig. at P = 0.01.
*** Sig. at P = 0.001.
[a] Percent citric acid.

its resemblance to normal conditions of testing, the evaluation is a memory comparison based on previous experience with similar stimuli. The paired presentation limited the evaluation to the 2 samples at hand and was considered more sensitive. This sensitivity was further increased when one member of the pair was kept constant within a set. Scaling of degree of difference between pairs yielded additional information, which could be plotted against the concentration of the additives.

ACKNOWLEDGMENTS

Appreciation is extended to the members of the taste panel for their interest and cooperation. The technical assistance of Mrs. Ida M. Trabue and Mrs. Sandra Gee is gratefully acknowledged. This investigation was supported in part by the Sugar Research Foundation, Inc., New York.

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Table 5. Analysis of variance of paired presentation, series II, method of constant stimulus.

Source of variation	Sums of squares	Degrees of freedom	Mean square	F ratio
Total	3,145.304	539		
Citric (C)	575.941	3	191.980	73.303***
Sucrose (S)	127.971	4	31.993	12.216***
Replications (R)	15.660	2	7.830	2.990
Judges (J)	826.904	8	103.363	39.467***
C × S	36.355	12	3.030	1.157
C × R	6.323	6	1.054	0.402
C × J	203.792	24	8.491	3.242***
S × R	36.117	8	4.515	1.724
S × J	150.429	32	4.701	1.795**
R × J	55.540	16	3.471	1.325
Remainder	1,110.272	424	2.619	

*** Sig. at P = 0.001.
** Sig. at P = 0.01.

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French-fried Potatoes: Palatability as Related to Microscopic Structure of Frozen Par-fries

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(Manuscript received June 7, 1960)

SUMMARY

When tissue was taken from finish-fried or par-fried potatoes not subjected to fixing or embedding, potato cells were found to be plump, and the surfaces were comparatively smooth, faintly showing the outlines of the gelatinized starch granules within. Cells from tissue that had been frozen showed reticulation, especially those from tissue frozen at −18°C as contrasted with tissue frozen at −78°C. When tissue was observed during freezing, cell separation was pronounced, as well as pushing together or shrinking of gelled starch granules, and cell distortion. When thawed, the potato cells partially recovered their original plumpness although breaks between the cells remained. Recovery of gelation was incomplete in starch granules from cells from potato tissue that had been par-fried, frozen, and finish-fried. A taste-test panel preferred French-fried potatoes frozen as an intermediate step in preparation, especially noting less cohesiveness of texture and greater tenderness of crust.

Preliminary studies in this laboratory indicated that French-fried potatoes prepared by par-frying, freezing, and finish-frying had better texture than similar potatoes French-fried without an intermediate freezing step in their preparation. This observation was the background for the work herein reported: a study of the microscopic appearance of fried potato tissue associated with freezing treatments and taste-panel scores.

During cooking of starchy vegetables, gelatinization of starch granules is one of the most noticeable phenomena. When moist heat is employed, vegetable tissues soften. Simpson and Halliday (1941) noted disintegration of cell membrane materials, softening of cellulose, and the changing of large amounts of protopectin to pectin. Reeve (1953) pointed out, among other histological observations on heated potatoes, that "cell separation, due to weakening of the adhesive properties of the middle lamellae, is aided by the swelling of gelled starch within the cells so that their walls are distended and pushed apart." Under some conditions, cells ruptured. He indicated that this is "caused by swelling of the gelled starch and appears to begin in the weaker primary pit regions of the cell walls."

Deep-fat frying might be assumed to have somewhat different effects, since in the frying process partial dehydration occurs. Reeve and Neel (1960) stated that "deep-fat frying is essentially a cooking and dehydration process during which the starch content of the cells is gelled and dehydrated and some of the water in the tissue is replaced with oil." They point out that the cellular structure remains intact and that the cell walls rarely rupture during the deep-fat frying process.

When cooked vegetable tissue is frozen, ice formation begins on the outside of the cells. If freezing occurs slowly, water is withdrawn from the cells and larger ice crystals are formed, which crush the cells (Gardner, 1940). Woodroof (1938) reported that in immature seeds such as peas, corn, and lima beans, cell walls separated and ruptured during freezing, while the contents of each cell coagulated into a shrunken mass. If the seed coats were uninjured, there was little loss of structure even though cell walls within the seed were broken or separated. Longree (1950), using mashed cooked potato, noted variations in cell appearance associated with rate of freezing. When shrunken cells were allowed to stand at room temperature or in a refrigerator, no reabsorption

took place of the water that had "frozen out" of the cells and starch granules. On reheating, the differences in cell appearance due to rate of freezing tended to disappear. Lee *et al.* (1946) tested the effect of freezing rates on the eating quality, appearance, and vitamin values of peas and snap beans. Although slower freezing rates had a pronounced effect on the appearance of the tissues by favoring the formation of large crystals and partially shrinking and dehydrating the solids, thawing caused the fluids to be reabsorbed by the tissues so that they resumed their natural appearance.

MATERIALS AND METHODS

Russet Burbank potatoes were obtained at harvest time from farms selected at random in commercial potato-growing areas. Tubers of high specific gravity, 1.115, and of lower specific gravity, 1.085, were selected from each farm in order to provide a means of determining specific-gravity effects.

Four methods for preparing finish-fried potatoes were compared: single-stage fried, unfrozen; two-stage fried, unfrozen; par-fried, frozen at −78°C, held 2 weeks on dry ice, then finish-fried; and par-fried, frozen at −18°C, held 2 weeks at this temperature, then finish-fried. Eight samples were

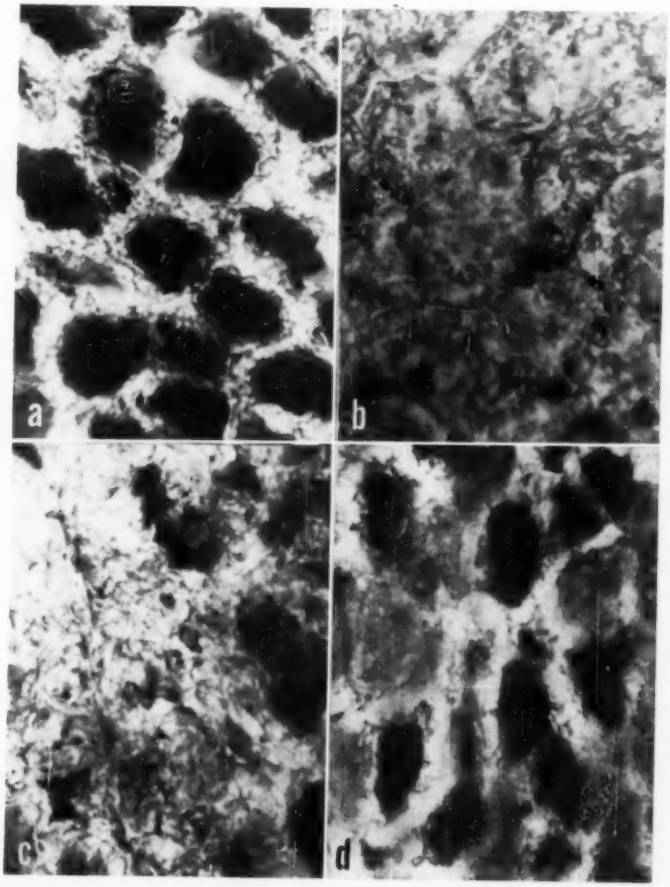

Fig. 1. Photomicrographs (×60) of potato tissue prepared for microscopic study by histological freeze-drying technique: a) finish-fried, unfrozen; b) par-fried, frozen at −18°C; c) par-fried, frozen at −78°C; d) par-fried, frozen at −18°C and finish-fried.

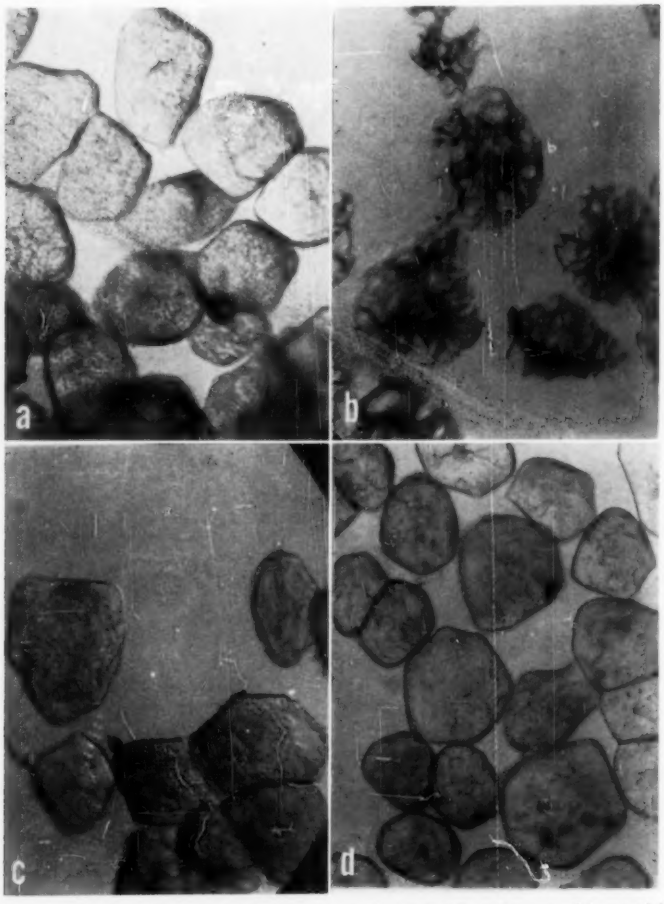

Fig. 2. Photomicrographs (×60) of potato cells: a) finish-fried, unfrozen; b) par-fried, frozen at −18°C; c) par-fried, frozen at −78°C; d) par-fried, frozen at −18°C and finish-fried.

evaluated per replication, and three replications were carried out.

Temperature during par-frying and finish-frying was maintained at 177°C. All par-fries were fried for 5 minutes. When finish-fried, the potato strips all had a total cooking time of 6½ min. Moisture loss and fat absorption were determined. All treatments were judged by a trained panel. Matching tubers from the same farms and of the same specific gravities were used for microscopic study.

Several procedures were used in preparing material for microscopic study, with the expectation that more information could thus be obtained. The histological freeze-drying technique (Sci. Specialties Corp., 1955) was selected in preference to the use of dehydrating solutions since tissue that is frozen very rapidly and dehydrated without thawing before infiltration with paraffin might be expected to bear a closer resemblance to untreated tissue. In addition, small bits of tissue were isolated from potato strips following par-frying, freezing at −18°C as well as −78°C, and after finish-frying. Bits of par-fried tissue were viewed during the processes of freezing, thawing, and refreezing.

The significance of differences due to specific gravity and treatment were determined by analysis of variance for panel scores, moisture loss, and fat absorption.

RESULTS AND DISCUSSION

Microscopic observations. The appearance of potato tissue differed somewhat with

the histological method used in its study. However, the effects of cooking and freezing were apparent although tissue was prepared by different methods.

Cells taken from potato strips prepared by par-frying or single-stage or two-stage frying without an intermediate freezing step were similar in appearance. When viewed by polarized light, no ungelatinized starch granules were found. Tissue taken from these strips was composed of plump cells crowded together (Fig. 2). Cell surfaces were faintly reticulated, having an over-all stippled appearance. Longree (1950) suggested that this effect is due to pushing of the swollen starch granules against the cell walls. Reeve (1943), in calling attention to this effect in sections cut from blanched, dehydrated potato after rehydration, wrote, "the semi-fibrous or reticulate appearance of the gelled starch may be due to variations in density as the grains swell or to cytoplasmic substances trapped between the swollen and gelled grains."

Ice crystals in cells frozen at −78°C must have been small enough that cells taken from par-fried tissue frozen and held for two weeks at this temperature closely resembled cells from tissue that had not been frozen. Evidence of strain from the freezing treatment was noted, in that surfaces of cells from frozen tissue were slightly wrinkled, in contrast to the velvety "stippled" appearance of the surfaces of unfrozen cells. The striations occurring in the cell surfaces from frozen tissue frequently had a twisted appearance (Fig. 2). The cells taken from strips that were par-fried and then frozen and stored at −18°C were generally similar in dimension and shape to those taken from unfrozen strips having no freezing treatment or having a "fast-freeze" treatment, except that the cell walls were very wrinkled, apparently adjusting to the contours of shrunken starch granules within (Fig. 2).

When par-fried tissue that had been frozen was finish-fried, the cells were plump and relatively smooth, probably from reabsorption of moisture and swelling of starch granules. Moisture loss was high during the par-frying operation (Table 2), but enough remained to allow the cells to become almost

Table 1. Average scores[a] for textural characteristics of French-fried potatoes prepared by various methods (scoring range: low, 1; to high, 4).

Characteristic	Treatment								LSD between means (P = .05)	
	Single-stage fried		Two-stage fried		Par-fried, frozen at −18°C & finish fried		Par-fried, frozen at −78°C & finish fried			
	S.G.[b] 1.115	S.G. 1.085	S.G. 1.115	S.G. 1.085	S.G. 1.115	S.G. 1.085	S.G. 1.115	S.G. 1.085	Treatment	Specific gravity
Over-all quality (preference)	2.2	2.4	2.8	2.6	2.9	2.7	3.6	2.9	.55	Not sig.
Crust crispness (crisp vs. lacking crispness)	2.5	2.5	3.3	2.7	3.2	3.1	3.5	3.5	.57	Not sig.
Crust tenderness (tender vs. tough)	1.9	2.7	2.5	3.0	3.3	3.7	3.6	3.8	.62	.43
Interior appearance (opaque vs. translucent)	3.1	2.3	3.3	2.8	3.1	2.4	3.6	2.5	Not sig.	.39
Interior graininess (granules, abundant vs. indetectable)	3.3	2.8	3.5	2.9	3.1	2.2	3.7	2.8	Not sig.	.36
Interior moistness (dry vs. soggy)	3.0	2.5	3.1	2.6	2.9	2.3	3.5	2.4	Not sig.	.27
Interior cohesiveness (particles disperse readily vs. cohesive)	2.9	2.7	3.3	2.6	3.4	2.9	3.6	3.3	.26	.18

[a] Each number is the average of 12 scores (4 judges, and 3 replications).
[b] S.G., specific gravity of tubers.

Table 2. Moisture loss and fat absorption during French-frying.

Sample	Specific gravity	Treatment	Moisture loss (%), fresh-weight basis		Fat absorption (%), finish-fried basis	
			Par-fry	Finish-fry	Par-fry	Finish-fry
1	1.085	Frozen at −18°C	43.6	47.5	5.8	8.1
2	1.085	Frozen at −18°C	44.2	46.9	7.0	8.5
3	1.115	Frozen at −18°C	43.3	46.5	7.3	8.7
4	1.115	Frozen at −18°C	41.5	44.3	5.2	7.1
5	1.085	Frozen at −78°C	43.9	46.4	6.6	9.9
6	1.085	Frozen at −78°C	41.9	44.3	5.6	9.2
7	1.115	Frozen at −78°C	44.7	47.1	7.9	10.8
8	1.115	Frozen at −78°C	45.4	47.7	7.9	11.7
9	1.085	Single fried	.....	48.5	.....	6.5
10	1.085	Single fried	.....	46.5	.....	6.8
11	1.115	Single fried	.....	46.5	.....	6.1
12	1.115	Single fried	.....	47.6	.....	8.4
13	1.085	Two-stage fried	.....	50.8	.....	9.3
14	1.085	Two-stage fried	.....	50.1	.....	8.2
15	1.115	Two-stage fried	.....	49.1	.....	8.7
16	1.115	Two-stage fried	.....	48.2	.....	7.6

fully restored to their original plump condition when finish-fried. That they were not completely restored is indicated by the tiny folds remaining in the cell walls (Fig. 2). According to Longree (1950), cooked potato cells that have been frozen in such a way that they become shrunken and reticulated, will regain their original plump, smooth appearance when heated in the presence of sufficient moisture.

Extensive shrinking of cells was apparent only when small bits of tissue were taken from par-fried potato strips and observed during the process of freezing. When handled in this way, cells originally plump and comparatively smooth changed in shape, becoming angular and wrinkled. Distortion of cooked cells, as well as cell separation during freezing, may be seen in photomicrographs (Fig. 3). Breaks in intercellular cementing material were marked. When cells were allowed to thaw, some reversal of the freezing change took place, the most noticeable being a plumping and partial recovery of the shape of the cells. Breaks between cells remained relatively prominent.

The freeze-drying method was used to secure information that might not be available from studies of single cells or small groups of cells isolated from cooked or cooked and frozen potato tissue. The cells in tissue prepared by the freeze-drying method did in fact differ in appearance from cells prepared for study in other ways. Because the freeze-dried and embedded tissue could be sectioned, the interior of many cells was visible.

A high percentage of water was lost during par-frying, and a small additional loss occurred during finish-frying (Table 2). In spite of the resultant partial dehydration, swelling and gelatinization of starch granules appeared to be unimpaired when no freezing step was included in the preparation. The cross sections of par-fried strips was similar in appearance to that of strips finish-fried in a single-stage or two-stage operation. The cells were well-filled with gelatinized starch granules (Fig. 1).

The cross sections of par-fries that had been frozen for two weeks had a porous appearance, indicating the growth of ice crystals throughout the tissue (Fig. 1). Dehydration of the gelled starch may be inferred, since granules that had been swollen during cooking to the extent that their outlines were obliterated and they filled the cells, appeared separate and distinct from each other after frozen storage. When finish-fried after two weeks of frozen storage at −18°C, cross sections of the potato strip showed cells incompletely filled with gelatinized starch granules (Fig. 1). Since cell walls are reported to be more hydrophilic than starch granules (Reeve, 1943), it is probable that

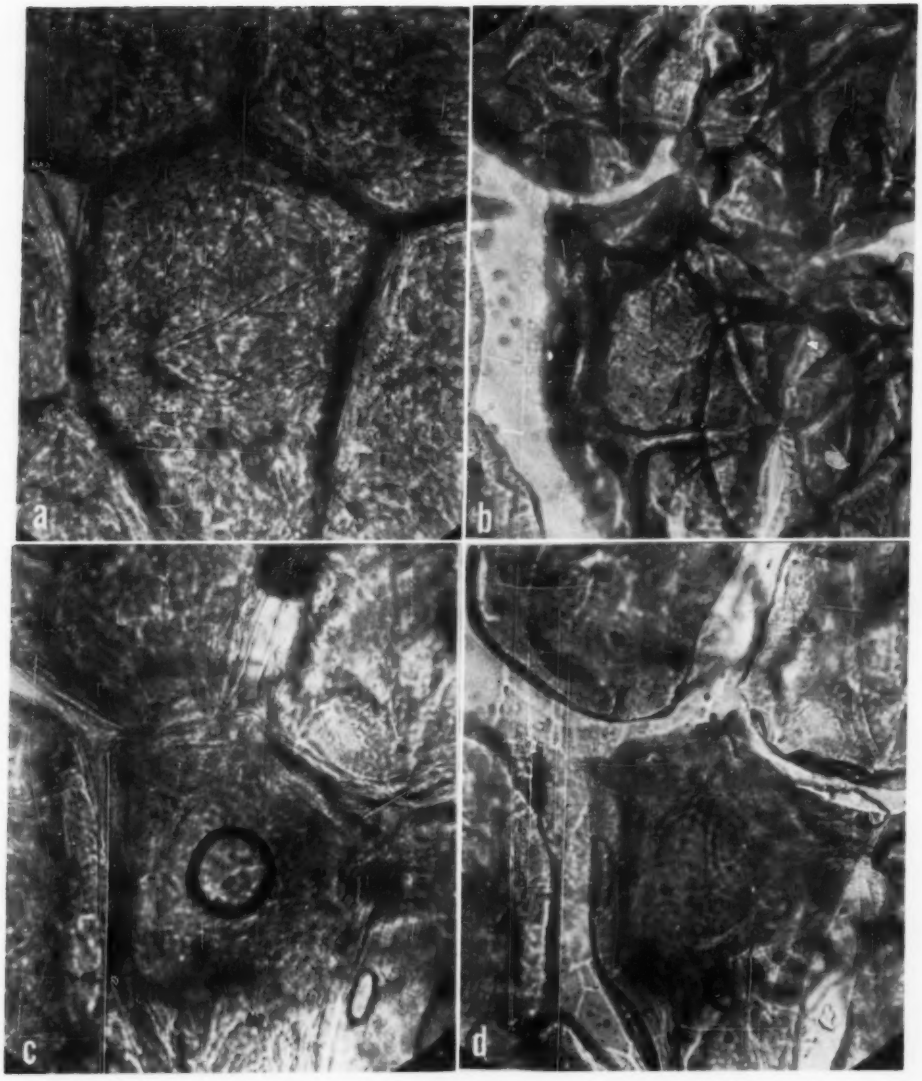

Fig. 3. Photomicrographs (×270) of finish-fried potato cells: a) unfrozen; b) frozen; c) thawed; d) re-frozen.

in the presence of a comparatively low percentage of water, the short cooking time after freezing was not sufficient to allow full recovery of the dehydrated starch granules. Swelling and gelatinization of starch was greater in tissues stored for two weeks at −78°C followed by finish-frying. In fact, this tissue closely resembled that having no freezing treatment. Cell separation was noted in cross sections of tissue that was finish-fried after freezing.

Panel scores. Table 1 shows average scores and significance of difference due to treatment for the characteristics of French-

fried potatoes prepared by different methods. Over-all quality, crust crispness, crust tenderness, and interior cohesiveness were influenced by treatment. The judges preferred the French-fried potatoes that had been par-fried, frozen, and finish-fried. According to their evaluation, the interior texture was less cohesive and the crust was more tender and crisp than in French-fried potatoes that had no freezing treatment.

High specific gravity of the tubers resulted in a more glistening, opaque interior, greater graininess, drier and fluffier interior, and less cohesive interior than was found for tubers of lower specific gravity. Although these differences in texture were noted by the taste panel, no consistent differences in microscopic appearance were found in par-fried or finish-fried tubers of different specific gravity classes.

Probably the most significant relation between microscopic appearance and taste-panel scores was the rupture of intercellular cementing material in the frozen par-fries, which would help account for the "noncohesive" texture scored high by the judges.

Moisture loss and fat absorption. Differences due to treatment were significant at the 1% level for moisture loss and fat absorption during frying to the finished stage. The potato strips that were frozen as an intermediate step in preparation absorbed more fat and lost less moisture than did the French-fries that were not frozen. The crisp, tender crust of French-fried potatoes that had been frozen and then finish fried was probably due to the slightly higher fat absorption by these potatoes.

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Influence of Autoclaving Soybean Proteins on Liberation of Aspartic and Glutamic Acids and Lysine by Several Digestion Procedures[a]

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(Manuscript received October 21, 1960)

SUMMARY

Part of the lysine was destroyed when soybean meal or isolated soybean protein mixed with sucrose was autoclaved 4 hr at 121°C. Little or no loss occurred when isolated proteins were autoclaved by themselves.

***In vitro* digestion with trypsin and erepsin liberated less lysine from the autoclaved than from the unheated soybean proteins if they did not contain the trypsin inhibitor.**

Partial hydrolysis with hydrochloric acid liberated more aspartic and glutamic acids and lysine from unheated than from heated soybean proteins.

Hydrolysis with concentrated hydrochloric acid for 7 days at 40°C and *in vitro* digestion with trypsin and erepsin liberated similar amounts of lysine (except for the proteins containing trypsin inhibitors), but acid hydrolysis liberated more aspartic and glutamic acids that did enzymatic hydrolysis.

The data support the hypothesis that autoclaving soybean protein formed lysine–aspartic acid and lysine–glutamic acid linkages that were resistant to mild hydrolysis.

Previous investigations have shown that overheating soybean oil meal reduced the protein nutritive value for growing chicks (Evans and McGinnis, 1946), and that the reduction was corrected by supplementation with lysine, methionine, and cystine, but not by any one of them alone (McGinnis and Evans, 1947). Autoclaving destroyed 43% of the lysine in soybean oil meal (microbiological assay after acid hydrolysis) and inactivated 60% (microbiological assay after enzymatic digestion) (Evans and Butts, 1948). No lysine was destroyed or inactivated by dry-heating soybean meal 4 hr at 121°C. None was destroyed when isolated soybean (Alpha) protein was autoclaved, but 30% was inactivated.

Block *et al.* (1946) postulated, for dry-heated cake mix, a reaction of the free carboxyl groups of the dicarboxylic amino acids with the epsilon-amino groups of lysine to form a new peptide linkage that would resist enzymatic digestion but be broken during acid hydrolysis. Lockhart and Abraham (1956) observed such a linkage in bacitracin A. Evans *et al.* (1951) observed that autoclaving 10 g of soybean (Alpha) protein for 4 hr at 121°C inactivated 1.24m*M* of basic amino acids (lysine, histidine, and arginine) and 1.25m*M* of acidic amino acids (aspartic and glutamic).

The work reported herein was conducted to see if lysine is both destroyed and bound in a form not released by enzymatic digestion when proteins other than Alpha protein are autoclaved, to determine how autoclaving proteins affects release of aspartic and glutamic acids and lysine by several digestion procedures, and to study further the nature of the lysine linkage resistant to enzymatic digestion.

EXPERIMENTAL

The materials used, percent nitrogen, and sources were: a commercial soybean oil meal (7.90%); Alpha protein (14.39%) and Amisoy (13.51%), both Glidden Company (now Central Soya Co.); Buckeye protein (14.32%), Buckeye Cellulose Corp.; C-I Assay protein (13.75%), Dracket Co. (now Archer-Daniels-Midland Co.); Protosoy

[a] Michigan Agricultural Experiment Station Journal Article No. 2696.

(13.64%), Mann Research Laboratory; casein (13.46%), zein (14.96%), gluten (14.08%), and lactalbumin (11.79%), all from Nutritional Biochemicals Corp.; and egg white (13.23%). The egg white was prepared by air-drying whites of eggs and grinding fine in a Wiley Mill.

Samples of soybean meal, proteins, or proteins plus 20% sucrose were heated 4 hr at 121°C in the autoclave at 15 lb pressure. Except where otherwise indicated, acid hydrolyses were performed by autoclaving 8 hr at 121°C with 20% hydrochloric acid. Enzymatic digests were prepared as described by Evans (1946). In some cases samples were hydrolyzed by standing several days in concentrated hydrochloric acid at 40°C.

Aspartic acid and lysine were determined by microbiological assay with *Leuconostoc mesenteroides* P-60 on the media of Sauberlich and Baumann (1946). *Lactobacillis arabinosus* was used to assay for glutamic acid.

Amino nitrogen was determined by the Van Slyke method. Amino acids were determined on some samples by the chromatographic procedure of Moore and Stein (1951).

RESULTS AND DISCUSSION

In agreement with earlier findings (Evans and Butts, 1951), 42% of the lysine in soybean oil meal, but none in Alpha protein, was destroyed by autoclaving (Table 1). When another isolated soybean protein, Buckeye protein, was autoclaved 14% of the lysine was destroyed (Table 1). The amount of lysine made available to *L. mesenteroides* by trypsin and erepsin digestion was nearly doubled when Buckeye protein was autoclaved 1 hr at 121°C (Fig. 1). Lysine availability in C-1 Assay protein, Amisoy, and Protosoy was also increased by autoclaving, but not nearly as much as in Buckeye protein (Fig. 1). Evans and McGinnis (1946) observed that chicks fed soybean flakes autoclaved 30 min at 121°C grew twice as fast as chicks fed unheated soybean flakes. The poor growth on raw soybean meal has been partially attributed to a trypsin inhibitor that was destroyed or inactivated by heating (Bowman, 1944; Ham and Sandstedt, 1944). Buckeye protein also probably contained the trypsin inhibitor, because the heated protein was more readily digested by trypsin than the unheated protein.

Ten per cent of the lysine was destroyed when casein was autoclaved, and 64% when casein plus sucrose was autoclaved (Table 1). Destruction of lysine was insignificant when zein or zein plus sucrose was autoclaved. The epsilon amino group of lysine in zein appears to be shielded in some way to prevent reaction with carbohydrate and tryptic release of lysine, because very little lysine (6% of total) was liberated from zein by enzymatic digestion *in vitro*.

Enzymatic digestion of Alpha protein, gluten, and lactalbumin released less aspartic and glutamic acids and lysine from autoclaved than unheated proteins (Table 1).

Because of the possible presence of trypsin inhibitor in some isolated soybean proteins, such as Buckeye protein, a non-enzymatic mild digestion procedure was needed to study the resistant linkage of lysine. Sanger and

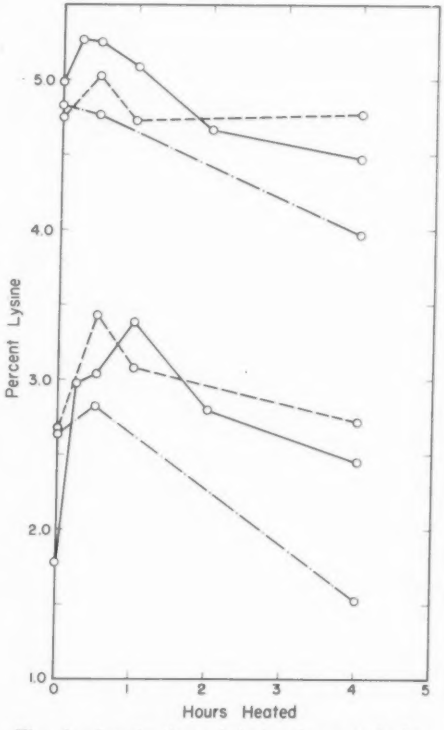

Fig. 1. Inactivation of lysine by autoclaving. Microbiological assay. Top curves: hydrolyzed 8 hr with 20% hydrochloric acid. Bottom curves: hydrolyzed with trypsin and erepsin. O———O: Buckeye protein. O—·—·—O: C-1 assay protein. O-----O-----O: Protosoy or Amisoy.

Table 1. Heat inactivation (%) of lysine, aspartic acid, and glutamic acid in some proteins.

Protein	Lysine				Aspartic acid				Glutamic acid			
	Acid hydrolyzed		Enzyme hydrolyzed		Acid hydrolyzed		Enzyme hydrolyzed		Acid hydrolyzed		Enzyme hydrolyzed	
	% [a]	Loss [b]	%	Loss	%	Loss	%	Loss	%	Loss	%	Loss
Soybean oil meal	2.90	42	1.28	94								
Alpha protein	4.48	0	2.95	45	10.72	5	3.52	47	16.72	1	6.05	38
+ sucrose	4.36	47										
Buckeye protein	4.79	14	1.52	−76	10.40	12	3.60	−18	21.42	−6	4.83	−68
+ sucrose	4.17	56										
Casein	6.32	10	5.42	25	6.69	9	1.94	−34	30.80	23	8.44	−23
+ sucrose	6.21	64										
Zein	1.02	−1										
+ sucrose	1.25	2										
Egg white	5.51	30	0.73	29	9.39	17	1.95	−2	13.37	52	3.55	50
Gluten	1.45	14	0.79	67	2.94	12	1.31	55	35.38	2	12.40	39
Lactalbumin	12.40	63	3.64	89	8.60	27	3.99	72	13.91	−9	8.83	89
C-1 assay protein	4.83	18	2.64	42	10.28	14	2.40	−2	15.84	5	3.38	16

[a] Percent on a dry-matter basis.

[b] Percent less of amino acid in autoclaved than in raw protein. A minus loss means that there was more amino acid in the autoclaved than in the raw material.

Tuppy (1951) partially hydrolyzed the phenylalanyl chain of insulin with 11*N* hydrochloric acid for 3 days at 37°C. The lysine-containing peptides of unheated and autoclaved Alpha protein were studied in the present experiment by hydrolyzing the proteins with concentrated hydrochloric acid at 40°C for from 8 hr to 7 days or with 20% hydrochloric acid at 121°C for from 15 min to 8 hr. The amount of lysine released in a form available to *L. mesenteroides* increased with digestion time, and lysine was released faster from unheated than from autoclaved proteins (Fig. 2). Thirty-seven percent less

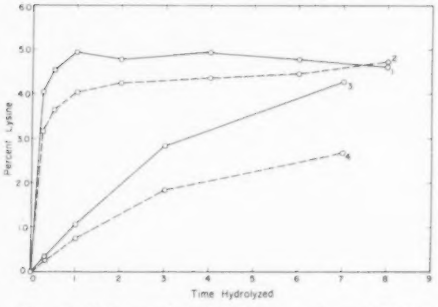

Fig. 2 Release of lysine from soybean (Alpha) protein in a microbiological available form by acid hydrolysis. 1) unheated protein hydrolyzed with 20% hydrochloric acid at 121°C; time in hours. 2) autoclaved protein hydrolyzed with 20% hydrochloric acid at 121°C; time in hours. 3) unheated protein hydrolyzed with concentrated hydrochloric acid at 40°C; time in days. 4) autoclaved protein hydrolyzed with concentrated hydrochloric acid at 40°C; time in days.

lysine was released from autoclaved than from unheated protein by hydrolysis with concentrated hydrochloric acid for 7 days and 42% less was released by enzymatic digestion.

The formation when soybean protein is autoclaved of a lysine linkage that resists hydrolysis is indicated by the above data. Lockhart and Abraham (1956), after hydrolysis of bacitracin A for from 21 to 91 hr with 11*N* hydrochloric acid at 80°C, isolated one peptide containing isoleucine, lysine, and aspartic acid, and another more stable one containing lysine and aspartic acid. Results with DNP-derivatives indicated that aspartic acid was linked to the epsilon-amino group of lysine. Evans *et al.* (1951) postulated that when soybean protein is autoclaved the free carboxyl groups of aspartic acid and glutamic acid react with the free amino groups of lysine and arginine and with the imidazole group of histidine to form a peptide-type linkage resistant to enzymatic cleavage. Several commercially isolated soybean proteins were studied to determine if equivalent amounts of dicarboxylic acids and lysine would be inactivated by autoclaving the proteins. Amino acid availability was determined by enzymatic and mild acid digestion.

Soybean oil meal, Amisoy, Buckeye protein, C-1 Assay protein, and Protosoy were hydrolyzed by enzymatic and acid digestion procedures to study the release of amino nitrogen, aspartic and glutamic acids, and lysine.

Hydrolysis of Alpha protein with concentrated hydrochloric acid for 7 days at 40°C liberated about the same amount of microbiologically available lysine but much more aspartic acid and glutamic acid than did digestion with a combination of trypsin and erepsin *in vitro* (Table 2). However, enzymatic digestion liberated slightly less lysine from autoclaved protein than acid digestion. Pepsin, trypsin, and erepsin digestion released more of all amino acids than trypsin and erepsin digestion. Increasing the time of acid hydrolysis to 14 days at 40°C and to 8 hours at 121°C increased amino acid release. Higher values for amino nitrogen, lysine, and aspartic acid but not for glutamic acid were obtained by chromatographic determination than by microbiological assay. Less lysine and aspartic acid were released from autoclaved than raw Alpha protein by enzymatic digestion or hydrolysis by concentrated hydrochloric acid at 40°C. Acid hydrolysis at 121°C released equal amounts of amino acids from raw or autoclaved proteins. Data for Amisoy, Buckeye protein, C-1 Assay protein, and Protosoy are not presented in tabular form because for the most part they are similar to those for Alpha proteins. The following differences were observed, however.

Table 2. Liberation of amino acids from Alpha protein (percent amino nitrogen or amino acid).

Amino acid	Total	Pepsin	Trypsin	Trypsin erepsin	Pepsin trypsin erepsin	Conc. HCl—40°C 3 days	Conc. HCl—40°C 7 days	Conc. HCl—40°C 14 days	20% HCl—121°C 30 min	20% HCl—121°C 8 hr
Amino groups	14.39[a]	1.99	3.98	4.90	4.52	5.86	7.14	7.67	9.02	11.44
Autoclaved	14.37[a]	1.51	3.77	4.51	5.13	5.34	6.87	7.55	9.10	11.28
% loss	0	24	5	8	−14	9	4	2	−1	1
Lysine	5.45[b]	0	1.77	3.73	4.36	2.84	3.86	4.49	3.82	4.86
Autoclaved	4.76[b]	0	1.00	2.39	3.64	2.04	2.93	3.36	3.23	4.68
% loss	13		44	36	17	28	24	25	15	4
Aspartic acid	12.70[b]	0.70	0.87	2.06	3.94	3.22	4.47	5.95	9.04	10.81
Autoclaved	11.06[b]	0.88	0.42	1.44	4.25	2.67	3.68	5.00	7.86	10.72
% loss	13	−26	52	30	−8	17	18	16	13	1
Glutamic acid	19.18[b]	4.00	5.30	4.43	6.24	9.92	11.86	13.52	17.87	20.74
Autoclaved	18.84[b]	2.66	3.52	1.96	6.66	10.20	11.87	13.32	17.27	21.29
% loss	2	34	34	56	−7	−3	0	2	3	−3

[a] Total nitrogen by Kjeldahl method.
[b] Chromatographic method.

Table 3. Lysine, aspartic acid, and glutamic acid made unavailable by autoclaving. (Millimoles per 10 g of protein).[a]

	Aspartic	Glutamic	Aspartic + glutamic	Lysine	Lysine, 20% HCl[b]
Trypsin-erepsin digestion					
Alpha protein	.47	1.68	2.15	.92	
Amisoy	+.57[c]	+1.26[c]	+1.83[c]	.21	
Buckeye protein	.04	.20	.24	.01	
C-1 Assay protein	.41	.73	1.14	1.46	
Protosoy	.39	.57	.96	1.33	
Conc. HCl 40°C 7 days					
Alpha protein	.59	.00	.59	.64	.12
Amisoy	.40	.52	.92	.85	.16
Buckeye protein	.56	+.22[c]	.56[d]	1.03	.40
C-1 Assay protein	.44	.10	.54	.88	.36
Protosoy	.55	.00	.55	.96	.29

[a] The millimoles of each amino acid made unavailable to the assay organism by autoclaving the protein were calculated by subtracting the amino acid content of the digest of autoclaved protein from the amino acid content of the digest of the raw protein to give g of amino acid per 10 g protein, which was divided by the millimolecular weight of the amino acid.
[b] Lysine unavailable after digestion with 20% hydrochloric acid for 8 hr at 121°C. This lysine was presumably destroyed by the Maillard reaction with carbohydrates.
[c] A + sign before the number means that autoclaving increased the availability of the amino acid.
[d] The glutamic acid value was not used in calculating the total dicarboxylic acids, because it increased in availability.

More free alpha-amino nitrogen, aspartic acid, and glutamic acid were released from autoclaved than from unheated Amisoy by trypsin or trypsin and erepsin, indicating Amisoy contained the soybean trypsin inhibitor. Lysine linkages resistant to mild acid digestion were formed when Amisoy was autoclaved, but destruction of amino acids was not significant.

Buckeye protein was affected by autoclaving essentially in the same way as Amisoy.

Autoclaving destroyed 10% of the lysine in C-1 Assay protein and 8% of that in Protosoy. Otherwise, autoclaving affected these two proteins in the same way as it did Alpha protein. Thus Alpha protein, C-1 Assay protein, or Protosoy could be used to study the nature of the lysine linkage resistant to enzymatic digestion, and any one of the five proteins could be used to study the nature of the acid-resistant lysine linkage.

Soybean oil meal contained 7.90% nitrogen (50% crude protein). No other constituents were determined, but soybean oil meal is usually about 22% carbohydrates (6% sucrose), 28% moisture, oil, ash, and fiber, and little or no reducing sugar (Peck, 1939). The constituent in soybean oil meal responsible for the destruction of lysine, appears to be sucrose, which is probably hydrolyzed to release reducing sugars, which in turn react with the epsilon-amino group of lysine by the Maillard Reaction. Autoclaving of soybean oil meal resulted in a greater decrease of alpha-amino nitrogen liberated by enzymatic digestion than did autoclaving of any of the isolated soybean proteins. Evans *et al.* (1951) observed that some protein-bound amino acids of soybean protein react with sucrose or glucose to form a linkage resistant to enzymatic but not to acid digestion.

In vitro digestion of 10 g of Alpha protein with trypsin and erepsin released 0.92m*M* less lysine, 0.47m*M* less aspartic acid, and 1.68m*M* less glutamic acid from autoclaved than from the unheated protein (Table 3). These data are not directly comparable with those of Evans *et al.* (1951) because no histidine or arginine determinations were made. Digestion with concentrated hydrochloric acid for 7 days at 40°C broke some of the linkages that enzymatic digestion did not, because the mild acid digestion released 0.64m*M* less lysine and 0.59m*M* less aspartic acid from the autoclaved than from the unheated protein. Similar amounts of glutamic acid were released. The indications from data with Alpha protein and Amisoy are that autoclaving of the protein caused formation of a linkage between lysine and aspartic acid and glutamic acid that was resistant to mild acid hydrolysis, because the m*M* of lysine and of aspartic plus glutamic acids released were nearly the same (Table 3).

The presence of the trypsin inhibitor in Amisoy and Buckeye protein interfered with enzymatic digestion studies on these proteins.

Buckeye protein, C-1 assay protein, and Protosoy appeared to contain sufficient carbohydrate so that part of the lysine was destroyed by the Maillard reaction. When the m*M* of lysine destroyed are subtracted from the total m*M* inactivated for these proteins, the m*M* of lysine made unavailable agree well with the m*M* of aspartic plus glutamic acids also made unavailable (Table 3).

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An All-glass Laboratory Apparatus for Concentrating Volatile Compounds from Dilute Aqueous Solutions

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(Manuscript received May 15, 1961)

SUMMARY

An all-glass apparatus for the concentration of volatile compounds from dilute aqueous solutions on a laboratory scale is described. 3–6 L of solutions are stripped in 1 hr under reduced pressure at temperatures at or below 37°C, and concentration increases of over 10 times are obtained in a single pass. A scale drawing of the apparatus and some examples of its operation are given.

The concentration of a great number of the odorous volatile compounds in the aqueous phase of food is on the order of parts per million or less. The concentration must be increased before fractional distillation, extraction, or other isolation procedures can be applied. The initial concentration increase obtained with any procedure should preferably be some 10 times or more, but may have to be reduced, depending on the properties of the compounds in the solution. No heat damage can be allowed, and care should be taken in this respect, particularly when nonvolatile material is also present in the liquid.

A number of devices for stripping the volatile compounds from dilute solutions have been described (Dimick and Makower, 1951; Kepner and Webb, 1956; Scott, 1956), of which the all-glass climbing film evaporator of Scott resembles in some aspects the apparatus described herein. One of the most advanced, producing excellent results, is the apparatus developed by Eskew *et al.* (1959), consisting of a stripper and a fractional distillation column in a combined arrangement. The method, with modifications, is used by many investigators but requires an elaborate set-up, which is not easily built in smaller laboratories and is more practical for large-scale operations.

All-glass rotating film evaporators are easy to use and to build for laboratory work (Kohn, 1956), but are designed primarily, and better suited, for concentrating nonvolatiles. Furthermore, their speed of operation is low (ca. 300/ml/hr at non-elevated temperatures).

In laboratory-scale concentrators for volatiles of the fractional distillation type as described for either atmospheric (Dimick and Simone, 1952) or for reduced pressure (Shepherd, 1957), the liquid from which the volatiles are to be recovered is temporarily heated to rather high temperatures (100 and 80°C, respectively). Such an apparatus is therefore more satisfactory for second-step concentrators where heat damage is less likely.

Recently, an arrangement (Cotner *et al.*, 1960) was described for the stripping of odors, with or without steam, which seems to have some advantages over a number of earlier steam strippers. No performance data are given.

The all-glass apparatus described here (Fig. 1) can be built easily and is simple to operate on a laboratory scale. The functional design was suggested by the stripper employed by Eskew and co-workers, but the construction utilizes some of the main structural features of a commercial apparatus (Jena-Schott, 1960) developed for the concentration of nonvolatile compounds.

APPARATUS AND OPERATION

When a vacuum (e.g., water jet) is established throughout the apparatus and the jacket of the heat exchanger A is well heated by steam from B, the

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solution to be stripped is sucked in at C. By regulating the stream with a stopcock or needle valve at D, the concentration increase can be chosen to some degree. As long as sufficient water is left unevaporated, the temperature is determined by the pressure in the system and is indicated by the thermometer at E.

The vapors developed in the heat exchanger are condensed in the condensor F and collected in the ice-cooled receptacle G. A small part of the highly volatile compounds will be found in the −80°C trap H. The residual liquid is recovered in the 5-L suction flask K, connected by way of an additional −80°C trap M to the vacuum source at N. The size of the suction flask K determines the amount of solution that can be stripped in one pass. By minor alterations, however, the apparatus can be made to operate continuously for any sample size.

PERFORMANCE

In one hour, 3–6 L of an aqueous solution, depending on its properties and on the concentration increase desired, can be run through the apparatus. Examples of solutions that were stripped without difficulties at 43–46 mm Hg and 36–37°C product temperature are: an aqueous solution containing 10 ppm of acetone and 10 ppm of ethyl acetate, apple juice, coffee brew, and beer from which part of the CO_2 was removed by standing 10 hours at +4°C. The increase in the concentration of volatile components for a single pass, determined by comparing gas-liquid chromatograms of vapor samples before and after stripping, was found to be between 10 and 30 times, depending on the product and its composition.

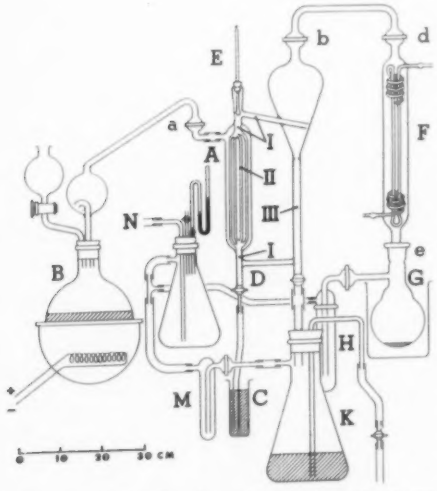

Fig. 1. Scale drawing of stripping apparatus. Outside diam. I, 10 mm; II, 4 mm; III, 11 mm. Not shown is the exit for condensed steam at the bottom of the heat exchanger A.

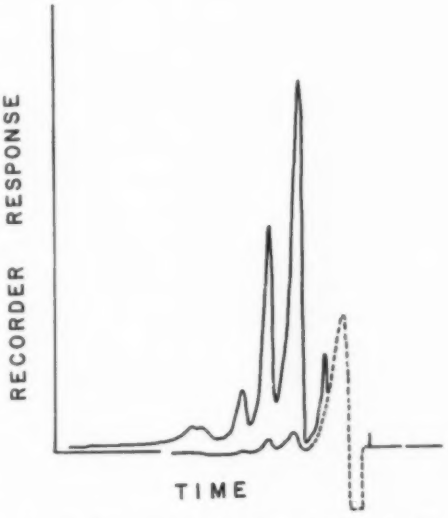

Fig. 2. Gas-liquid chromatograms of headspace vapors over commercial pasteurized milk (bottom graph) and its single-pass concentrate (top graph). Column 130 × 0.6 cm; 10% di-*n*-decylphthalate on firebrick, 80–100-mesh, at 50°C; Sr^{90} ionization detector at 1750 V; 95 ml argon/min. Sample size, 5 ml.

Re-stripping of the first concentrate from the synthetic mixture gave total respective concentration increases of 119 and 126 times over the initial concentration of the acetone and the ethyl acetate.

The odor of the concentrates in all applications was very much strengthened and of excellent quality as compared with the original solutions.

Normally, the apparatus is not recommended for stripping milk with a high fat content. The odor of the concentrate obtained from such milk, however, was excellent, and the increase in concentration of the volatiles was *ca.* 18 times on the first pass, but the narrow tubes in the heat exchanger tended to clog and required cleaning after 4 L had been stripped in 40 min at a maximum temperature of 38°C. The results of the milk-stripping test are demonstrated in Fig. 2 by two superimposed gas-liquid chromatograms of samples of headspace vapors taken over 25 ml of concentrate and of the corresponding original liquid in 250-ml Erlenmeyer flasks at room temperature. The increase in con-

centration can easily be read from the two graphs, since the peak heights have been found to be directly proportional to the concentration of the volatile compounds in solution (Weurman, 1961).

ACKNOWLEDGMENT

The development and testing of the apparatus has been part of a research program at the Quartermaster Research and Engineering Command, Natick, Mass., during a Research Associateship awarded by the National Academy of Sciences—National Research Council. The help of Mr. Willard T. Hall in constructing the apparatus is gratefully acknowledged.

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The search for Truth is in one way hard and in another easy. For it is evident that no one can master it fully nor miss it wholly. But each adds a little to our knowledge of Nature, and from all the facts assembled there arises a certain grandeur.

—ARISTOTLE